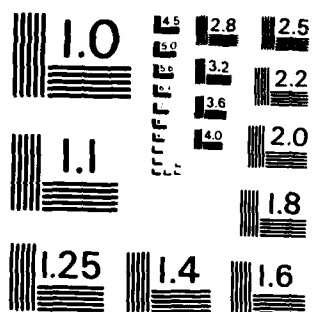END
DATE
FILMED

DTIC

MICROCOPY RESOLUTION TEST CHART

NATIONAL BUREAU OF STANDARDS-1963-A

AD_____

EFFECTS OF TRYPANOCIDAL DRUGS ON THE FUNCTION OF TRYPANOSOMES

Annual Progress Report

George C. Hill

September 1978

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-74-C-4046

Colorado State University
Fort Collins, Colorado 80523

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution limited

83 08 03 119

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. $A131010$	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Effects of Trypanocidal Drugs on the Function of Trypanosomes		5. TYPE OF REPORT & PERIOD COVERED Annual Progress Report Jan. 1, 1978 - Sept. 1, 1978
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) George C. Hill		8. CONTRACT OR GRANT NUMBER(s) DAMD -17-74-C-4046
9. PERFORMING ORGANIZATION NAME AND ADDRESS Colorado State University Department of Pathology Fort Collins, CO 80523		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62770A 3M162770A802.00.051
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE 1 September, 1978
		13. NUMBER OF PAGES 88
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

Same as report

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Cultured-infective trypomastigotes; T. rhodesiense; T. brucei; berenil; suramin; L-α-GP-oxidase; macromolecular synthesis.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The purpose of our studies has been to determine the effects of trypanocidal drugs on the function of trypanosomes. We have also been interested in determining the mode of action of trypanocidal drugs. Our approach to resolving this problem includes investigating various enzymes in host and trypanosomes, studying the effects of trypanocidal drugs on enzyme systems isolated from trypanosomes and studying the structure and transcription ability of purified kinetoplast DNA. We are interested in determining the reason for the unique selective toxicity of known trypanocidal drugs.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 68 IS OBSOLETE
S/N 0102-014-6601 |

Our primary results during this past year are:

a.) Established two strains of <u>Trypanosoma</u> <u>brucei</u> (<u>T. brucei</u> EATRO 110, and <u>T. brucei</u> 427) <u>in</u> <u>vitro</u> as cultured infective trypomastigotes (1);

b. Established <u>T. rhodesiense</u> EATRO 1895 <u>in</u> <u>vitro</u> as cultured infective trypomastigotes (2);

c. Established <u>T. equiperdum</u> ATCC 30023, a naturally occuring dyskinetoplastic trypanosome, <u>in</u> <u>vitro</u> as culture infective trypomastigotes;

d. Measured the inhibition of RNA and protein synthesis in <u>T. brucei</u> treated <u>in</u> <u>vivo</u> with berenil and suramin, and

f. Isolated purified nuclear and kinetoplast DNA from <u>T. rhodesiense</u> for hybridization with nuclear and mitochondrial RNA.

TABLE OF CONTENTS

ABSTRACT

The purpose of our studies has been to determine the effects of trypanocidal drugs on the function of trypanosomes. We have also been interested in determining the mode of action of trypanocidal drugs. Our approach to resolving this problem includes investigating various enzymes in trypanosomes, studying the effects of trypanocidal drugs on enzyme systems isolated from trypanosomes and studying the structure and transcription ability of purified kinetoplast DNA. We are interested in determining the reason for the unique selective toxicity of known trypanocidal drugs.

Our primary results during this last year are:

a. Established two strains of Trypanosoma brucei (T. brucei EATRO 110, and T. brucei 427) in vitro as cultured infective trypomastigotes (1);

b. Established T. rhodesiense EATRO 1895 in vitro as cultured infective trypomastigotes (2);

c. Established T. equiperdum ATCC 30023, a naturally occurring dyskinetoplastic trypanosome, in vitro as cultured infective trypomastigotes;

d. Measured the inhibition of RNA and protein synthesis in T. brucei treated in vivo with berenil and suramin;

e. Measured reduced berenil and increase αGP oxidase activity in T. brucei treated in vivo with suramin;

f. Isolated purified nuclear and kinetoplast DNA from T. rhodesiense for hybridization with nuclear and mitochondrial RNA.

APPROACH TO THE PROBLEM

Our approach to the problem of developing effective trypanocidal drugs is to study two specific and related areas of the biochemistry of trypanosomes, nuclear and mitochondrial gene expression. The interrelationships of these two systems can be seen in Figure 1. In addition, we are concerned with the regulation and control of the functioning of the terminal oxidases in bloodstream trypomastigotes. In order to identify targets for potential trypanocides, we must learn more about the properties of the mitochondrion in trypanosomes including the replication and transcription of KDNA and the repression and synthesis of mitochondrial electron transport systems. We also need to study the properties of the α-GP oxidase system. If we can alter the functioning of the mitochondrion or other essential electron transport systems in trypanosomes, we should be able to inhibit the continuation of the life cycle of the trypanosome.

We are also interested in the inhibition of novel enzymes on processes which are under control of the nuclear genome. These include processes such as antigenic variation, synthesis of L-α-GP oxidase synthesis of cytoplasmic ribosomes or other enzymes or processes. Our goal in the development of novel trypanocidal drugs is to gain additional knowledge on the control mechanisms involved in the transcription of nuclear and mitochondrial genes in these organisms. In this way, we hope to identify potential targets for trypanocides that so far have not been investigated. The proper establishment of the primary mode of action of a drug requires a systematic study of its effect on the various metabolic processes of the cell at the lowest concentrations that inhibit growth. Only when such a survey is

complete can it be concluded confidently that a particular pathway or reaction is most sensitive to inhibition by a drug and is therefore the primary target of that drug. We are carrying out this type of systematic study with several trypanocidal drugs including berenil, suramin, antrycide and ethidium bromide. These types of studies have only rarely been performed with trypanocidal drugs.

The numerous and necessarily speculative points raised in any discussion of trypanocidal drug action are now mostly capable of experimental verification with currently available techniques and data from related fields of cell biochemistry. Given the necessary attention, this largely neglected but important field should yield results of considerable value, not only for an understanding both of trypanocidal drug action and of trypanosomal metabolism, but for cell biology in general. This is the purpose of this contract. So far as trypanocidal drug design is concerned, the era of intelligent empericism is unlikely to be superseded until the balance of effort and expenditure on drug production is adjusted more favorably in the direction of research on the metabolism of trypanosomes and on the mechanisms whereby existing drugs exert their specific effects.

In developing new trypanocidal agents, we are investigating:

1. The effects of trypanocidal drugs affecting enzyme systems in trypanosomes in hosts;

2. Detailed comparisons of homologous enzymes in host and trypanosomes;

3. Unique cell components or metabolic pathways in trypanosomes;

4. The basis of the selective toxicity of known drugs.

THE BACKGROUND

African trypanosomiasis is confined to Africa by the distribution of its vectors. T. gambiense infection occurs over a broad belt from Senegal in the west to the great lakes Victoria, Albert, Benguelo, and Tanzania in the east, extending as far south as north Angola. T. rhodesiense is located in east and central Africa and is scattered over a longitudinal band from the south Sudan to Mozambique. The importance of trypanosomiasis of man and animals in Africa has been summarized recently by Kershaw (3) in the statement:

> "The World Health Organization in determining the ten
>
> major health problems facing mankind places trypanosomiasis
>
> of man and his domestic animals high on the list along with
>
> malaria, cancer and heart diseases."

Identification of New Trypanocidal Drugs

The need for new trypanocides cannot be overemphasized. At present, chemotherapy of African trypanosomiasis is dependent on a relatively small number of synthetic drugs. Suramin and pentamidine are used for prophylaxia and treatment of early stages of the disease in man. Organic arsenicals such as tryparsamide and melaminyl compounds are used for advanced cases, when trypanosomes have invaded the central nervous system. The disease in cattle and other domestic animals is controlled by quaternary ammonium trypanocides (e.g., antrycide, ethidium, prothidium, and related drugs) and by the aromatic diamidine, berenil. As pointed out recently by Newton (4), resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to another.

In human trypanosomiasis, there is still an urgent requirement for a cheap, simply administered and well-tolerated, preferably "one-shot" drug which would be as effective a prophylactic as pentamidine and active therapeutically against all stages of the infection in both Gambian and Rhodesian sleeping sickness. It should also be incapable of inducing drug resistance and active also against strains with acquired resistance to other drugs.

Possibly the two requirements of prolonged tissue retention (for prophylaxis) and ability to penetrate into the central nervous system are mutually exclusive, but with increasing knowledge of the structure and function of the so-called "blood-brain barrier," this problem should not be insuperable.

In none of the active drugs is the mode of action precisely known. An excellent review on the mode of action of trypanocidal drugs has been prepared by Williamson (5). More recent studies have suggested that berenil and ethidium bromide form complexes with DNA. In the case of ethidium, it is clear this drug is a potent and selective inhibitor of DNA synthesis. It has been shown by several investigators that both phenanthridines and acridines combine with DNA by the heterocyclic chromophore of the drug mole .les becoming inserted, or intercalated, between the adjacent base pairs in the double-stranded helix of DNA. Such intercalation is achieved by a partial uncoiling of the DNA helix which results in the base pairs above and below the bound drug molecule becoming separated by twice their normal distance (4).

More recently, it has been shown that phenanthridines also bind to supercoiled DNA of the type found in certain tumor viruses, mitochondria

of many cell types and kinetoplast of trypanosomes. There is evidence that these drugs bind preferentially to such DNA in vivo and give rise to dyskinetoplastic trypanosomes (6) and "petite mutants" of yeast (7). The molecular basis of this preferential binding is not yet fully understood. The findings that have been observed could adequately explain the growth inhibitory activity of phenanthridine drugs but it remains to be established whether their primary action on bloodstream forms of trypanosomes is to inhibit DNA synthesis.

Berenil, an aromatic diamidine, has been shown to interact with DNA and can selectively block kinetoplast replication (8, 9). The earliest reported effect observed of berenil is the localization in the kinetoplast of T. brucei. This has been detected by ultraviolet microscopy with an hour of a curative dose being injected intraperitoneally into infected mice and within seconds of the drug being added to an in vitro suspension of trypanosomes (9). Recent work has shown that berenil can form complexes with purified DNA, but in contrast to phenanthridines, there is good evidence that the complexes are not formed by intercalation (10).

A detailed examination of kinetoplast DNA isolated from berenil-treated T. cruzi has shown that many of the small circular DNA molecules appear as branched structures (8). These forms, which are thought to be replicative molecules, are rarely seen in control preparations, suggesting that berenil does not block the replication of kinetoplast DNA at initiation but binds preferentially to certain specific points in the circular DNA molecule. As for phenanthridines, it cannot be said what is the primary effect of erenil or other diamidines on trypanosomes.

The mode of action of suramin remains enigmatic even after more than a half century of use. In vitro exposure to trypanosomes to suramin at concentrations as low as 10^{-5} M is known to reduce their infectivity whereas concentrations as high as 10^{-2} M do not affect the motility or respiration of cells. As would be expected from its structure, the drug binds avidly to basic proteins and is known to inhibit many isolated enzymes (5). The most sensitive enzymes examined appear to be hyaluronidase, inhibited at 10^{-5}-10^{-6} M, fumarase, inhibited at ca. 10^{-7} M, urease at pH 5 (ca. 10^{-4} M), hexokinase (10^{-4}-10^{-5} M), and RNA polymerase (10^{-5} M) (11). Recent studies by our laboratory supported by this contract (12) and other investigators (13, 14) have demonstrated that suramin also inhibits the L-α-GP oxidase in bloodstream trypanosome in vitro. Whether this is its mode of action in vivo remains to be determined.

The ready absorption of this drug by plasma proteins may well account for the long retention time of the compound in man and animals and contribute to its value as a prophylactic agent. The question of how a molecule as large as suramin enters trypanosomes is an interesting one and it seems possible that, when protein bound, suramin actively stimulates pinocytosis. As with the other drugs that we have discussed, there is evidence that suramin becomes localized in lysosomes. Again whether this is important to the trypanocidal action of the drug or whether it is a secondary phenomenon is unknown.

The Importance of African Trypanosomiasis as a Public Health Problem

Human trypanosomiasis, causing sleeping sickness, and animal trypanosomiasis, affecting cattle and other domesticated animals, are the two classical notorious plagues of Africa rooted in the continent since time immemorial.

Sleeping sickness constitutes a permanent and serious risk to the health and well-being of at least 35 million people, and animal trypanosomiasis is the main obstacle to the development of the vast potential for livestock production in the continent. Any involvement of our military troops in areas endemic with African trypanosomiasis would be disastrous.

Ten thousand new cases of human trypanosomiasis are known to occur yearly, but this figure does not truly reflect the importance of the disease as a public health problem. As for many tropical diseases, prevalence figures are underestimates due to failure to recognize the disease and to under-reporting. The relatively low prevalence is due to the major control efforts which have been made over the past 50 years. Without these, sleeping sickness would still be a major cause of death, as it was at the turn of the century, with great epidemics raging along the Congo river and the northern shores of Lake Victoria costing the lives of some 750,000 people.

At the present time, some 10 million people at risk are examined annually by mobile teams at an estimated cost of 5 million dollars. Expenditure for control of tsetse flies is at least ten times higher, but the total is difficult to evaluate since most efforts are directed towards control related to animal disease.

In view of the potential serious danger of sleeping sickness, national health services accord high priority to control services but efforts are frequently inadequate since sufficient resources in terms of finance, manpower and administrative facilities may not be available.

An outbreak of sleeping sickness is a dramatic event in a community since the disease causes severe symptoms due to lesions of the central nervous system and is fatal if not treated. Outbreaks may cause populations to abandon villages and fertile farmlands, and the effect is such that even after two generations, fear of re-exposure may prevent the people from returning.

Current control measures do not usually eliminate the disease; moreover, they are costly and cumbersome. With the available tools, control is a continuing effort, producing suppression rather than eradication. The experience of the past 50 years has been that whenever control efforts are interrupted, for example due to political or economic circumstances, or out of complacency, a flare-up of the disease will sooner or later occur.

A recent example was the resurgence of trypanosomiasis in Zaire in the early 1960's, when after six years of interruption of surveillance, prevalence figures rose from 0.01% to 12%, and even to 18% in some areas. Outbreaks of unknown severity are now reported from Angola, Cameroon and the Sudan. It is to be expected that more outbreaks will occur in coming years unless improved control measures can be found.

Development of new tools is therefore a matter of urgency, not only as a means to eradicate the disease but to provide measures which are more effective and can be more widely applied than those presently available.

RESULTS AND DISCUSSION

A. Cultivation of Trypanosomes *In Vitro*

During this past year, one of our primary goals has been to develop a culture system for T. brucei and T. rhodesiense cultured infective trypomastigotes. We have been successful in growing in vitro cultured infective forms of:

a. T. brucei 427

b. T. brucei EATRO 110

c. T. rhodesiense EATRO 1895

d. T. equiperdum ATCC 30023

All of the organisms have been initiated and maintained on Chinese hamster lung tissue culture cells. The results with T. rhodesiense have been discussed in a manuscript to be published shortly in Science. The results with T. brucei shall be published shortly in Acta Tropica. Preprints of the manuscripts are included in the appendix.

Using primarily T. brucei and T. rhodesiense, it has been possible for us to develop a culturing system which will be further refined as our research progresses. Infective forms of T. brucei and T. rhodesiense have been initiated and maintained in vitro at 37°C over Chinese hamster lung and buffalo lung tissue culture cells. In the following section, we shall discuss the progress made in the initiation, maintenance and characterization of this in vitro system.

Tissue Culture Cells

The tissue culture cell lines that we have used include buffalo (Bison bison) lung tissue culture cells (ATCC CCL 40, BV, IMR-31) or Chinese

hamster (Cricetulus griseus) lung tissue culture cells (ATCC CCL 16 Don).
As noted, both of these lines are available from the American Type Culture
Collection (ATCC), Rockville, MD. Thus, it is possible to use commercially
available tissue culture cell lines for maintaining the infective forms, a
distinct advantage over the procedures required for obtaining the bovine
fibroblast-like cell line used by Hirumi et al. (15).

The Chinese hamster lung and buffalo lung cell lines were maintained
in HEPES-buffered Rosewell Park Memorial Institute (RPMI) 1640 medium with
20% heat inactivated fetal bovine serum (FBS). Both of these components
were obtained from GIBCO, Grand Island, NY and K-C Laboratories, Kansas City,
MO respectively. The medium also contained per 100 ml, penicillin –
10,000 U, fungizone – 25 ug, streptomycin – 10 mg and kanomycin – 12.5 ug.
The morphology of both tissue cell lines was fibroblast-like and a descrip-
tion of the cell lines is available from ATCC. When maintained at $37^{\circ}C$
in an atmosphere of 5% CO_2-95% air, an inoculum of 1.8 x 10^6 viable tissue
culture cells multiplied 3-5 fold in 7 days. The tissue culture cells
were used just prior to becoming confluent, usually 3-4 days after a
flask was inoculated. Stabilates of both tissue culture cells have been
prepared in 10% glycerol.

Preparation of Trypanosomes for Culture

Successful cultures with T. brucei 427, T. brucei EATRO 110, or T.
rhodesiense EATRO 1895 were obtained using the following procedures for
the preparation of the trypanosomes. Irradiated rats (800 rads) were
inoculated with 2 x 10^6 trypanosomes. With both organisms, the cell
population was 90-95% slender forms. When the parasitemia reached a level
of 1-5 x 10^8 trypanosomes/ml three to four days later, the rat was bled

by cardiac puncture. The trypanosomes were centrifuged at 1025 g and the buffy coat removed. Special precautions were taken to prevent the removal of red blood cells below the buffy coat. The trypanosomes were washed twice with Hank's balanced salt solution with 5% sodium citrate. The trypanosomes were then inoculated into a total of 5.0 ml of medium (RPMI and 20% FBS containing the previously mentioned antibiotics) in T-25 flasks or 15.0 ml in T-75 flasks. Most successful experiments occurred in those initiated in the T-75 flasks.

Initiation of *T. brucei* Cultures

An experiment with $\underline{T}$. brucei was performed by inoculating a T-75 flask of buffalo lung cells with an initial concentration of 8×10^4 trypanosomes/ml. The trypanosomes were incubated at $37^{\circ}C$ in an atmosphere of 5% CO_2 - 95% air in the medium described. Two days later, a third of the medium was changed. The trypanosomes remained at a low count, less than 1×10^5 cells/ml for the next four days. The cell numbers then began to increase and on day 8 reached a cell count of 5×10^5 cells/ml. One-third of the medium was then changed daily and on day 11, the cell count was 2.5×10^6 cells/ml. By changing one-third of the medium daily, the cells have been maintained subsequently at a concentration of $2-4 \times 10^6$ cells/ml. In recent experiments, it is clear that 5% CO_2 is not necessary in order for the trypanosomes to grow. These results can be seen in Table I.

It was also possible to initiate flasks after incubation of the trypanosomes from irradiated rats in the medium at $25^{\circ}C$ for 24 hours prior to adding the trypanosomes to the tissue culture cells at $37^{\circ}C$. Cells maintained for up to three days at $25^{\circ}C$ in the described medium were as infective as

controls for mice and rats. In addition, 20-30% of the cells were observed

dividing on day 2 and day 3. These results were discussed in the Annual

Report provided 1 January, 1978.

Additional cultures from the original buffalo lung flask were initiated

on either buffalo lung cells or Chinese hamster lung cells. The flasks

were usually initiated at 2×10^5 cells/ml. The results of a typical experi-

ment on Chinese hamster lung cells are presented in Table II. On day 3 after

inoculation, with one-third of the cell medium changed daily the cell count

was 2.1×10^6 cells/ml, reflecting a population doubling time of 12 hours.

The daily trypanosome count for T. brucei 427 continued at $1.0-3.0 \times 10^6$

cells/ml until the tissue cells had to be changed. T. brucei EATRO 110 grows

slightly higher (Table III). The trypanosomes were changed to fresh tissue

culture cells every 7 days. However infective forms of trypanosomes were

maintained in older cultures for 20-30 days, depending on the age of tissue

culture cells.

Initiation of T. rhodesiense Cultures

T. rhodesiense was initiated in culture using similar procedures as

described for T. brucei. However, the trypanosomes were initiated on Chinese

hamster lung tissue culture cells. The trypanosome numbers remained low,

less than 1×10^5 cells/ml for 10 days. Then the numbers increased to 5×10^5

cells/ml on day 12. One-third of the medium was then changed and a new flask

started at 4×10^5 cells/ml. The flask then began to increase markedly. The

results of the growth of trypanosomes in a new flask initiated at this point

are given in Table IV. Under identical cultural conditions, T. rhodesiense

grows faster than T. brucei. With one-third of the medium changed daily T.

<u>rhodesiense</u> has been maintained in culture at 3-5 x 10^6 cells/ml. The
population doubling time for <u>T</u>. <u>rhodesiense</u> is 9-11 hours.

Growth of Trypanosomes *In Vitro*

The trypanosomes grow not only in the medium but also in spaces between
the tissue culture cells. In figure 2, one can observe <u>T</u>. <u>brucei</u> 427 on
buffalo lung tissue culture cells. Figure 3 provides similar results for
<u>T</u>. <u>brucei</u> EATRO 110 which often grows very close to the tissue culture cells.
With all strains of trypanosomes, organisms are often observed in the spaces
between the tissue cultrue cells. In addition, trypanosomes can be seen
swimming free in the medium. In the case of <u>T</u>. <u>rhodesiense</u>, the trypano-
somes also grow close to the tissue culture cells as well as free in the
medium (figures 4 and 5).

Morphology of Trypanosomes *In Vitro*

The morphology of trypomastigotes maintained by rapid passage at 24
hours intervals in cultures was identical to that of the long sledner forms
of <u>T</u>. <u>brucei</u> or <u>T</u>. <u>rhodesiense</u> observed in the bloodstream <u>in vivo</u>. Few
stumpy forms were observed and as can be seen in figures 6A and B, the
cultured trypanosomes had subterminal kinetoplasts. In addition, some
bizarre forms were observed in culture. The bizarre forms were particularly
present in cultures with older tissue culture cells and were also observed
by Hirumi <u>et</u> <u>al</u>. (15) in their cultures. As seen in Giemsa stain prepara-
tions, these forms appear to be multinucleate with many membranes present.
Often these forms were seen dividing.

The ultrastructural studies of the trypanosomes provided excellent
evidence that the cultured infective trypanosomes had a fine structure

similar to the bloodstream trypomastigotes. The ultrastructure of T. brucei is reported in the Acta Tropica paper in the appendix (1). See figures 1-3 in this manuscript. It was identical to T. rhodesiense which we shall discuss in more detail. The surface coat can be clearly seen on the T. rhodesiense cultured infective trypomastigotes (figures 7-10). It can be easily seen in figure 9 and 10. In figure 8, the mitochondrion with few cristae can be observed. This is a characteristic of salivarian slender bloodstream trypomastigotes. Figure 11 is an electron micrograph of a T. rhodesiense bloodstream trypomastigote. Note the presence of the thick surface coat. In addition, figures 12-14 are T. rhodesiense procyclic trypomastigotes with the surface coat absent.

It is clear from these studies that the T. rhodesiense cultured infective trypomastigotes have identical fine structure to the bloodstream trypomasti-gotes and are different from the procyclic trypomastigotes.

Terminal Respiration of Cultured Trypanosomes

Slender trypomastigotes have an α-GP oxidase which is sensitive to SHAM (figure 15). In order to provide further evidence that the cultured infective trypomastigotes were slender bloodstream trypomastigotes, we have studied the steady state oxidases in these cultured forms. Ninety ml of culture fluid of T. rhodesiense from inoculated Chinese hamster lung cell cultures were harvested at a concentration of 2.5×10^6 trypanosomes/ml. The organ-isms collected by centrifugation and the steady state oxygen levels observed. The results are given in figure 16. Salicylhydroxamic acid (SHAM) inhibited 90% of the cell respiration. Azide had little effect, inhibiting 7% of the cell respiration. Little significant azide and SHAM insensitive respiration

was observed. Similar results were obtained for cultured infective trypo-
mastigotes of T. brucei (1). These results provide evidence that the SHAM
sensitive α-GP oxidase is the predominant terminal oxidase in trypomastigotes
cultured in our system. In addition, we can distinguish cultured infective
trypomastigotes from procyclic trypomastigotes which have little SHAM-sensit-
tive respiration and a significant azide-sensitive respiration (figure 17).
These differences are clearly presented in Table IV.

Infectivity of Cultured Trypanosomes

Experiments to test the infectivity of the cultures of T. brucei or T.
rhodesiense were performed with stabiltaes of bloodstream trypomastigotes
as controls. The mice were the descendents of a four-way cross of four
inbred strains, DBA/2 CIJax, C57B1/6 and BALB/c. In one such experiment,
they were inoculated with 1×10^4, 1×10^5 and 1×10^6 T. brucei trypano-
somes which had been in culture for 20 days. The results of the experiment
are given in figure 18. Infectivity tests were also performed on non-irrad-
iated mice at day 17, 24, 30, 33, 40 and 44. The cultures all produced
patent infections. These cultures continue to grow and are infective to
mice after 80 days in culture.

In similar experiments, the cultures of T. rhodesiense have also
produced infections in mice. Mice infected with 1×10^6 cells produced
patent infections 4-5 days post-inoculation Table VI. These cultures cont-
inue to be infective to mice after 80 days in culture.

Studies on the infectivity of the cultured trypomastigotes have
demonstrated that all the organisms maintained in culture are infective
to mice and rats. These organisms include T. brucei 427, T. brucei EATRO

110, T. rhodesiense EATRO 1895 and T. equiperdum ATCC 30023. However,
several differences between the culture and bloodstream trypomastigotes
have been observed.

1. In all the organisms studied, during the first 20-25 days in
    culture, the time necessary to kill mice is the same as controls;

2. After the first three weeks, the cultured trypomastigotes develop
    parasitemia at the same time as control but require a longer period
    before the mice die. Low parasitemias are maintained for 20-40
    days and a large amount of variation occurs. However, T. brucei EATRO
    110 is an exception. After 50 days in culture, we have observed
    no difference in comparison to the controls.

3. All cultured organisms are infective to rats but the parasitemias
    of the cultured trypomastigotes are very low and rarely kill the
    host.

We now need to determine what is the reason for the reduced parasitemia
after growth in culture. This is an extremely important question and may
assist us in developing new knowledge against African trypanosomiasis.

B. **Effects of Various Drugs on Bloodstream Trypomastigotes**

Our efforts have been devoted to an examination of T. brucei treated in vivo with several trypanocidal drugs including suramin, ethidium bromide, berenil and antrycide. The criteria we have considered includes:

a. effects on fine structure;

b. effect on RNA and protein synthesis;

c. effect on continued development on parantemia;

d. effect on cell respiration.

Hadjuk (16) has written an excellent review on the influence of DNA complexing compounds on the kinetoplast of trypanosomatids. In the literature reviewed, there are numerous experiments using insect trypanosomatids or bloodstream organisms in vitro. Amazingly, there are few, if any, studies reported which study the biochemical effects of trypanocidal drugs of trypanosomes treated in vivo. A few studies have been performed on the ultra-structure of trypanosomes isolated from any drug-treated hosts (17, 18), but biochemical studies are lacking.

During the past year, we have continued these studies with T. brucei. In our experiments, after the parasitemia level reached a level of 5-10 x 10^8 trypanosomes/ml, the rats were treated at 10 mg/kg with trypanocidal drugs (e.g., antrycide, suramin, ethidium bromide and berenil). After the parasitemia had been reduced 20-30% by the various drugs, the trypanosomes were separated from the blood by centrifugation, washed with an isotonic buffer, and used in the biochemical experiments. Table VII provides the protocol for such an experiment. The fine structure, respiration and RNA and protein synthesis

information was obtained from such an experiment.

Cells treated with berenil (10 mg/kg), as observed in a tail vein blood smear, were markedly affected morphologically after only 15-20 minutes exposure to the drug. The trypanosomes became elongated and noticeably sluggish. When these trypanosomes were harvested, they had a distinct yellow color in contrast to the white pellet of the control. The ethidium bromide pellet had a faint pink appearance. All other pellets from drug treated cells appeared as the controls.

Effect on Fine Structure

In the ultrastructural studies, the trypanosome pellet was fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer. The organisms were post-fixed with 2% OsO_4, dehydrated and stained with lead acetate. A comparison of the ultrastructure of the cells in table VII provided information on the effects of trypanocidal drugs on the fine structure of the drug-treated trypanosomes. The berenil treated cells were markedly affected (figures 19-21). There was an effect on the kinetoplast DNA (figure 19) as well the nuclear DNA (figures 20 and 21). Figure 21 provides clear demonstration of the nucleus in various stages of degradation after treatment with berenil. These results and the fact that they are observed within 15 minutes strongly suggest the effect of berenil is not specific to KDNA. In addition, figure 22 demonstrates that the parasitemia after treatment with berenil is reduced dramatically after 2 hours, a result not likely if the effect of the drug were only on KDNA. The period required for decrease is less than the population doubling time of the trypanosomes (e.g., 4-6 hours) a mode of inhibition of berenil not specific for KDNA.

In the case of ethidium bromide, after exposure of the trypanosomes for two hours, no specific effect was observed. Both the kinetoplast DNA (figures 23 and 24) and nuclear DNA (figure 25) was affected. Antrycide has a marked effect on the population of ribosomes as well as the cytology of the cell (figure 26). Even though the parasitemia decreased after 12-14 hours exposure to suramin (figure 27), no effect on the ultrastructure of the trypanosomes was observed in comparison to the controls (figure 28).

Williamson and co workers (17, 18) have also studied the fine structure of trypanosomes exposed to trypanocidal drugs in vivo and observed several effects. They concluded that the kinetoplast is the principal focus of berenil action. Similar studies by these investigators have shown that curative dosages of antrycide on bloodstream trypanosomes cause the same type of KDNA condensation, after 5-6 hours, as seen in diamidine (berenil and hydroxystil-bamidine) treated cells (19, 20). These observations suggest that antrycide binds preferentially to the KDNA in vivo and alters the structural organization of the KDNA network.

Effects on RNA and Protein Synthesis-Exposure to Drugs In Vivo.

The effect of these trypanocidal on the synthesis of RNA and protein was also investigated. The trypanosomes were harvested as described in the previous experiment and then resuspended in RPMI medium with 5% FBS to 2×10^8 cells/ml in a total of 5 mls. The trypanosomes were then labelled with 25 mCi (5 mCi/ml of either or ^3H uridine for RNA synthesis or ^3H leucine for protein synthesis). Samples were taken at time points 0, 10, 20, 30, 40, 50 and 60 minutes. These were then precipitated and filtered with 5% TCA and read in the liquid scintillation counter for 10 minutes with 3% error. The

results are presented in tables VIII and IX and figures 22 and 23.

Berenil had a marked effect on RNA synthesis, inhibiting uridine in-corporation 87.2% ± 3.7 S.E. In addition, protein synthesis was inhibited 30.1% ± 2.3 S.E. Results with antrycide and ethidium bromide can also be seen in figures 29 and 30. Antrycide markedly inhibited protein synthesis (table IX). The other drug that was studied extensively was suramin. Treatment with suramin at 10 mg/kg for 12-13 hours under the same experimental procedures reveals a 2% decrease in the average leucine incorporation and 88.3% decrease in the average uridine incorporation. Our results show that suramin drastically inhibits the synthesis of RNA by the trypanosomes and has little effect on protein synthesis.

As previously mentioned, when bloodstream T. brucei is exposed in vivo to berenil (10 mg/kg), the trypanosomes become elongated in 15-30 minutes. This same effect has been noted in vitro when control bloodstream trypomastigotes are removed from the rat or cultured infective trypomastigotes are exposed to 5-10 μg/ml. In each case, the effect is observed in 80-90% of the cells 1-2 hours after the organisms are incubated at 37°C in RPMI 1640 medium plus 20% FBS. These results demonstrate some of the effects of berenil seen in vivo with T. brucei can be observed in vitro. No morphological effect has been observed in vitro with procyclic trypomastigotes. Experiments are now required to accurately measure the amount of berenil and ethidium bromide that binds to those organisms over a specific period of time.

Effects on RNA and Protein Synthesis-Exposure to Drugs *In Vitro*

Along with our studies on the effects of trypanocidal drugs on RNA and protein synthesis in trypanosomes, we have been investigating the effects of

various metabolic inhibitors (e.g., chloramphenicol, cyclohexamide, ethidium bromide and actinomycin D) on $\underline{T}$. $\underline{brucei}$. We have now identified media for excellent incorporation and growth of trypanosomes $\underline{in}$ $\underline{vitro}$, thus enabling an investigation of the RNA and protein synthesis $\underline{in}$ $\underline{vitro}$ to occur. The SM medium of Cunningham (21) is excellent for procyclic trypomastigotes and the RPMI medium is excellent for bloodstream trypomastigotes. Few studies have been reported under ideal conditions. In addition, because of the known mode of action of these inhibitors (figure 1), the response of both procyclic and bloodstream trypomastigotes is important.

Trypanosomes were incubated at 5×10^7/ml in 2.5 ml of medium. As noted, for bloodstream trypomastigotes, RPMI plus 5% fetal bovine serum (FBS) was used. For procyclic trypomastigotes, SM medium plus 5% FBS was used. The RNAs and proteins were labeled with ^{3}H uridine and ^{3}H leucine respectively. The RNAs or proteins synthesized were precipitated with TCA and counted.

Results with ethidium bromide provide a clear difference between procyclic and bloodstream trypomastigotes of $\underline{T}$. $\underline{brucei}$ EATRO 110. After 60 minutes incubation in the presence of ethidium bromide, procyclic trypomastigotes required three-fold more ethidium bromide in comparison to bloodstream trypomastigotes in order to inhibit 50% of the RNA synthesis. As seen in figure 31, bloodstream trypomastigotes are clearly more sensitive to ethidium bromide than procyclic trypomastigotes.

In order to determine the contribution of cytoplasmic and mitochondrial protein synthesis in $\underline{T}$. $\underline{brucei}$ procyclic and bloodstream trypomastigotes,

we have been investigating the inhibition by chloramphenicol and cyclo-
hexamide. In procyclic trypomastigotes, the maximum inhibition with
cyclohexamide is 85.6% at levels between 100-300 µg/ml (0.4 - 1.1 mM).
In contrast, chloramphenicol inhibits protein synthesis 18-22% at concentra-
tions of 100-200 ug/ml (0.31 - 0.62 mM). Concentrations of chloramphenicol
higher than 0.62 mM kill the trypanosomes. This concentration is considerably
less than 50 mM 914.1 mg/ml) concentration recommended for T. brucei by
Hanas et. al. (29). These results would suggest a significant amount of
chloramphenicol sensitive protein synthesis (18-22%) occurs in procyclic
trypomastigotes. In addition, with high concentrations of cyclohexamide
and chloramphenicol, 10% leucine incorporation continues to occur.

In bloodstream trypomastigotes, markedly different results occur.
High concentrations of cyclohexamide from 20-200 ug/ml (0.062 mM-0.6. mM)
inhibited leucine incorporation in most experiments only 35-40%. The
results may be due to the presence of the surface coat preventing penetra-
tion of the cyclohexamide. At low concentrations (0.062 mM), chloramphenicol
actually stimulated leucine incorporation 15%. At higher concentrations of
0.31-0.62 mM chloramphenicol, 18-25% inhibition of leucine incorporation
occurred, quite comparable to the results with procyclic trypomastigotes.
Concentration of cyclohexamide or chloramphenicol above 500 ug/ml killed
the bloodstream or procyclic trypomastigotes. Results with actinomycin also
show a greater sensitivity to this drug by bloodstream rather than procyclic
trypomastigotes (Table X).

Effects of Respiration

We have also studied the effects of suramin and berenil on the steady states and total respiration evident after the trypanosomes were isolated from drug-treated rats (Table VII). The purpose of these experiments was to gain additional information on the biochemical mode of action of these trypanocides. The results are presented in table XI. Antrycide and ethidium bromide have little effect on the respiration of the cells. However, suramin-treated cells had a marked increase in respiration. This is surprising since suramin is thought to inhibit the α-GP oxidase in trypanosomes. In contrast, berenil markedly inhibits the respiration of the organisms. This effect occurs as early as 1 hour after treatment with the drug. No difference was observed in the steady state level of various oxidases. Further studies in this area are needed, particularly in order to determine what respiratory enzymes are stimulated after treatment with suramin.

C. Determination of the Presence of an RNA Transcript.

Studies to identify an RNA transcript from kDNA from T. rhodesiense

have continued.

The steps included in this phase of research include the following:

a. Growth of 40 liters of T. rhodesiense;

b. Purification of network kinetoplast DNA;

c. Purification of Pst 1 restriction endonuclease;

d. Digestion of network kinetoplast DNA with Pst 1;

e. Purification of RNA's from T. rhodesiense;

f. Hybridization of RNA's to nuclear or kinetoplast DNA columns

    (figure 33).

Growth of T. rhodesiense

The laboratory strain of T. rhodesiense (EATRO 1895) has been used

and stabilated blood from infected animals has served as the primary source

for our investigations. This strain of T. rhodesiense was adapted for growth

in vitro. Tail vein blood was secured aseptically from rats infected with

stabilated organisms. One-hundred microliter drops of the blood were de-

posited into 2 ml of SM medium (21) and the mixture incubated for 3 days

at 27°C. The cells were completely transformed by day 4 and were then

diluted into 10 ml of fresh SM medium. The trypanosomes were maintained

in this medium by subculturing every 3 days to an initial concentration of 1

x 10^6 cells/ml. All of our experiments have been conducted with these or

similarly adapted cells of T. rhodesiense (EATRO 1895).

Amino acid analysis was performed on the SM medium before and after

T. rhodesiense had grown in it for 7 days (Table XII). Conversion of the

total picrate soluble amino acids to their n-trifluoroacetic acid n-butyl esters was carried out according to Gehrke (22) and analysis was performed by gas chromatography on an ethylene glycol adipate/OV-17: SP-2401 column. Our results were similar to those reported by Cross et al. (23) and Brun and Jenni (24) showing the relative increases in glutamine/glutamic acid, phenylalanine, methionine, serine, proline, glycine and valine. Subsequently, we have modified the SM medium by removing alanine and glycine and by decreasing the glutamine and proline concentrations by one-tenth those indicated by Cunningham (21). Growth of our cells in this modified media (SM*) was comparable to the original medium (SM) and is depicted in figure 32.

In order to isolate 300 mg of maxicircle DNA, we had to obtain 15-20 gm wet weight of T. rhodesiense cells, which meant harvesting 40 liters of growth media over a 5 week period. The cells were inoculated into 320 ml of sterile aliquots of SM* medium in Roux bottles at an initial concentration of 1×10^6 cells/ml (figure 32). After 2 days at 27°C, the trypanosomes were harvested. The average cell density was 7.0×10^6 cells/ml. The sedimented trypanosomes were resuspended in isotonic buffer and pelleted again. Liquid nitrogen was then used to rapidly freeze the washed pellets which were subsequently stored at -70°C.

Labelling and Isolation of T. rhodesiense RNA

The nucleic acids of T. rhodesiense were labelled with ^{32}P in order to aid in the detection, quantitation and characterization of the RNA fractions binding to the mitochondrial DNA-cellulose column. The labelled DNAs will be characterized as to their base composition and hybridized to

cold RNA fractions with sequence homology. One-hundred ml of SM* medium
was inoculated with actively growing cells to an initial concentration of
1×10^6 cells/ml. Five millicuries of ^{32}P were added aseptically and the
culture was incubated for 3 days at $27^{\circ}C$. The labelled cells were collected
by centrifugation at 6,000 rpm at $4^{\circ}C$. The pellets were washed in 25 ml
of cold buffer and centrifuged again. The final pellet was rapidly
frozen with liquid nitrogen.

The zonal centrifugation procedure of Glisin et al. (25) as modified
by Hallick et al. (26) for securing global RNA was adapted for use with
this system. Sarkosyl lysates of T. rhodesiense were treated with pronase
then adjusted to 1.609 g/ml with solid CsCl. The mixture was then centrifuged
over a pad of CsCl at 1.707 g/ml for 16 h at $20^{\circ}C$. Under these conditions,
the RNA and network kDNA sediments and nuclear DNA collects at the inter-
face of the two CsCl solutions. The inclusion of aurintricarboxylic acid
(AT) in the lytic buffer competitively inhibits nuclease activity.

Purification of Pst 1 Restriction Endonuclease

It has been shown by several investigators that the restriction endo-
nuclease Pst 1 frees maxicircles from network kDNA (27). The maxicircles
are thought to be the only kDNA component which is transcribed into RNA
and will be necessary in our hybridization experiments. Thus, Pst 1
restriction endonuclease is being purified in our laboratory by the modified
technique of Bickle et al. (28). The organism carrying this enzyme was
kindly provided for us by Dr. Julian Davis from the University of Wisconsin.
Providencia stuartii 164 (Pst 1) was grown overnight at $30^{\circ}C$ then inoculated
into 4 liters of L-Broth. The cells were harvested when they reached an
O.D. of 1.1 at 550 nm. The cell pellets were rapidly frozen in liquid

nitrogen and stored at $-70^{\circ}C$ until needed. Frozen pellets were thawed slowly at $4^{\circ}C$ in 2 x volumes of a Tris buffer, then sonicated for a total of 2 minutes at $4^{\circ}C$ using an output of 75 watts/10 sec. For the small volumes used, the temperature change after each burst was monitored and found to increase about 4 degrees. Cooling time in between sonic bursts was 6 mins. This allowed the temperature to return to $4^{\circ}C$ before another cycle was begun. The sonicate was clarified by centrifugation at 100,000 x g for 120 mins. The supernatant fractions were collected and adjusted to 1 M NaCl. This solution was than applied to a BioGel A 0.5 column for fractionation based on size.

A heparin-agarose column was prepared in our lab by activating BioGel A 1.5 with cyanogen bromide then adding excess amount of heparin (100 mg/50 ml settled volume). The dialyzed Pst 1 solution was applied to the column, equilibrated with 0.01 M phosphate buffer and eluted with a linear salt gradient (0.05/0.5 M NaCl) in the same buffer. The active fractions were again collected and dialyzed against phosphate buffer containing 0.2 M NaCl and 50% glycerol. The enzyme was stored at $-20^{\circ}C$ until needed.

Enzyme containing fractions were detected by their ability to hydrolyze DNA to specific size fragments. The DNA was purified in our laboratory from E. coli CSH 45 for this assay. Agarose gel electrophoresis was used to separate the fragments of DNA and demonstrate which fraction contained the restriction endonuclease. The enzyme solution was pooled, dialyzed and stored at $0^{\circ}C$ until it could be applied to the next column. With purified Pst 1, we will be able to proceed to isolate maxicircle DNA from T. rhodesiense and begin our hybridization studies.

CONCLUSIONS

Our primary results during this last year are:

a. Established two strains of <u>Trypanosoma</u> <u>brucei</u> (<u>T</u>. <u>brucei</u> EATRO 110,
    and <u>T</u>. <u>brucei</u> 427) <u>in</u> <u>vitro</u> as cultured infective trypomastigotes (1);

b. Established <u>T</u>. <u>rhodesiense</u> EATRO 1895 <u>in</u> <u>vitro</u> as cultured infective
    trypomastigotes (2);

c. Established <u>T</u>. <u>equiperdum</u> ATCC 30023, a naturally occuring dys-
    kinetoplastic trypanosome, <u>in</u> <u>vitro</u> as cultured infective trypomasti-
    gotes;

d. Measured the inhibition of RNA and protein synthesis of <u>T</u>. <u>brucei</u>
    treated <u>in</u> <u>vivo</u> with berenil and suramin;

e. Measured reduced berenil and increase αGP oxidase activity in <u>T</u>.
    brucei treated <u>in</u> <u>vivo</u> with suramin;

f. Isolated purified nuclear and kinetoplast DNA from <u>T</u>. <u>rhodesiense</u>
    for hybridization with nuclear and mitochondrial RNA.

RECOMMENDATIONS

Our recommendations would be the following:

1. Continue attention to the development of an in vitro system for growing

and maintaining cultured-infective trypomastigotes. Our next step should be

the establishment of cultures from single cell populations. This would be of

value in studying numerous processes in trypanosomes including drug resistance,

antigenic variation and nuclear and mitochondrial genes processes. In addition,

a long term goal is to grow the trypanosomes in the absence of the feeder cells;

2. Direct attention not only to mitochondrial gene processes but also gene

expression at the nuclear level. It is unlikely, that experiments devoted only

towards mitochondrial gene expression will resolve all chemotherapeutic efforts.

Thus, attention to processes controlled by nuclear genes would also be necessary

and extremely important;

3. Continue support for biochemical characterization of trypanosomes obtained

from drug-treated rats. This system lends itself to the isolation of trypano-

somes which have been treated in vivo with drugs and then can be analyzed

biochemically.

4. Novel approaches to the development of trypanocidal drugs are needed.

With the advent of new techniques in molecular biology, this should certainly

be possible. Some potential targets include the α-glycerophosphate oxidase.

Can we develop agents to present the synthesis of this important enzyme? We

already know the suramin inhibits its functions. Can we inhibit the synthesis

of this important oxidase as well enzymes of the anaerobic pathway? In this

regard, it will be necessary to purify the L-α-GP oxidase and identify the anaerobic pathway before we can begin to identify the genes and mRNAs responsible for their synthesis. This approach is considered in our renewal.

A three-part approach is required. Many more metabolic studies are needed to unravel the biochemical pathways that occur in parasitic protozoa and to identify further potential targets for chemotherapeutic attack. In addition, more research needs to be carried out on the biochemical modes of action of existing drugs and on mechanisms of resistance since such studies not only help to elucidate the metabolism of the organism but also provide information which will be vital to future attempt at more rational drug therapy directed towards specific target sites. As well as these biochemical studies it is essential that the conventional method of finding new drugs, the screening of compounds against parasites in vitro, or in animals, should continue. Information from the other two approaches, as it becomes available, should enable an increasingly more rational approach to be taken in the selection of compounds to be synthesized and tested.

References

1. Hill, G.C., Shimer, S., Caughey, B. and Sauer, S. 1978. Growth of infective forms of Trypanosoma (T.) brucei on buffalo lung and Chinese hamster lung tissue culture cells. Acta-Tropica - in press.

2. Hill, G.C., Shimer, S., Caughey, B. and Sauer, S. 1978. Growth of infective forms of Trypanosoma rhodesiense in vitro, the causative agent of African Trypanosomiasis. Science - in press.

3. Kershaw, W.E. 1970. Forward. In: The African Trypanosomiasis, pp. XII-IX. Mulligan, H.W. (ed.). (George Allen and Unwin Ltd., London).

4. Newton, B.A. 1974. Chemotherapy of trypanosomiasis and leishmaniasis. Trypanosomiasis and Leishmaniasis with Special Reference to Chagas Disease, pp. 285-307. (Associated Scientific Publishers, New York).

5. Williamson, J. 1970. Review of chemotherapeutic and chemoprophylactic agents. The African Trypanosomiasis, pp. 125-221. Mulligan, H.W. (ed.). (George Allen and Unwin Limited, London).

6. Steinert, M. 1969. Specific loss of kinetoplastic DNA in trypanosomatidae treated with ethidium bromide. Exp. Cell Res. 55: 248-252.

7. Slonimski, P.P., Perrodin, G. and Croft, J.H. 1968. Ethidium bromide induced mutation of yeast mitochondria. Biochem. Biophys. Res. Commun. 30: 232-239.

8. Brack, C.H., Delain, E., Riou, G., and Festy, B. 1972. Molecular organization of the kinetoplast DNA in Trypanosoma cruzi treated with berenil, a DNA interacting drug. J. Ultra. Res. 39: 568-579.

9. Newton, B.A. and Le Page, R.W.F. 1967. Preferential inhibition of extracellular deoxyribonucleic acid synthesis by the trypanocide berenil. Biochem. J. 105: 50.

10. Waring, M.J. 1970. Variation of the supercoils in closed circular DNA by binding of antibiotics and drugs: evidence for molecular models involving intercalation. J. Mol. Biol. 54: 247-279.

11. Hill, G.C. and Bonilla, C.A. 1974. In vitro transcription of kinetoplast and nuclear DNA in Kinetoplastida. J. Protozool. 21: 632-638.

12. Hill, G.C. 1977. Characterization of the electron transport systems present during differentiation of African trypanosomes. Function of Alternative Oxidases (ed. Degn, H., Lloyd, D. and Hill, G.C.) pp. 67-77, Pergamon Press, Oxford.

13. Evans, D.A., Brown, R.C. 1973. m-chlorobenzhydroxamic acid-an inhibitor of cyanide-insensitive respiration in Trypanosoma brucei. J. Protozool. 20: 157-160.

14. Clarkson, A., Brohn, F. 1976. Trypanosomiasis: an approach to chemotherapy by the inhibition of carbohydrate catabolism. Science 194: 204-206.

15. Hirumi, H., Doyle, J.J. and Hirumi, K. 1977. African trypanosomes: Cultivation of animal-infective Trypanosoma brucei in vitro. Science 196: 992-994.

16. Hadjuk, S.L. 1978. Influence of DNA complexing compounds on the kinetoplast of trypanosomatids. Prog. Mol. Subcellular Biol.. 6: 158-200.

17. MacAdam, R.F. and Williamson, J. 1969. Lesions in the fine structure of Trypanosoma rhodesiense specifically associated with drug treatment. Trans. Roy. Soc. Trop. Med. Hyg. 63: 421-422.

18. MacAdam, R.F. and Williamson, J. 1972. Drug effects on the fine structure of Trypanosoma rhodesiense: diamidines. Trans. Roy. Soc. Trop. Med. Hyg. 66: 897-904.

19. MacAdam, R.F. and Williamson, J. 1974. Drug effects on the fine structure of Trypanosoma rhodesiense. Ann. Trop. Med. Parsit. 68: 291-299.

20. Williamson, J., MacAdam, R.F. and Dixon, H. 1975. Drug-induced lesions in trypanosome fine structure: a guide to modes of trypanocidal action. Biochem. Pharmacol. 24: 147-151.

21. Cunningham, I. 1977. New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. J. Protozool. 24: 325-329.

22. Zumwalt, R.W., Roach, D. and Gehrke, C. 1971. Gas-liquid chromatography of amino acids in biological substances. J. Chromatography 53: 171-180.

23. Cross, G.A.M., Klein, R.A. and Linstead, D.J. 1975. Utilization of amino acids by Trypanosoma brucei in culture: L-threornine as a precursor for acetate. Parasitology 71: 311-326.

24. Brun, R. and Jenni, L. 1977. A new semi-defined medium for Trypanosoma brucei sspp. Acta-Tropica 34: 21-33.

25. Glisin, V., Crkvenjakov, R. and Byus, C. 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry 13(12): 2633-2637.

26. Hallick, R.B., Chelm, B.K, Gray, P.W. and Orozco, E.M., Jr. 1977. Use of aurin-tricarboxylic acid as an inhibitor of nucleases during nucleic acid isolation. Nucleic Acids Res. 4(9): 3055-3064.

27. Borst, P. and Fairlamb, A.H. 1976. DNA of parasites, with special reference to kinetoplast DNA. Biochemistry of Parasites and Host-Parasite Relationships (ed. Van den Bossche, H.) pp. 169-191). North Holland, Amsterdam.

28. Bickle, T.A., Pirrotta, V. and Imber, R., 1977. A simple, general procedure for purifying restriction and endonucleases. Nucleic Acids Research 4(8): 2561-2572.

29. Hanas, J., Linden, G. and Stuart, K. 1975. Mitochondrial and cytoplasmic ribosomes and their activity in blood and culture form Trypanosoma brucei. The Journal Cell Biology. 65: 103-111.

TABLE I

GROWTH OF T. BRUCEI IN PRESENCE AND ABSENCE OF CO_2

Day	Control [a] (trypanosomes/ml)	Plus CO_2 [b] (trypanosomes/ml)
0	1.1×10^5	1.0×10^5
1	6.8×10^5	2.9×10^6
2	1.6×10^6	2.0×10^6
3	5.3×10^6	2.4×10^6
4	3.6×10^6	1.4×10^6

a - One-third of the RPMI medium was changed daily in both flasks. The control flask was incubated in 100% air.

b - This flask was incubated in 95% air, 5% CO_2.

TABLE II

GROWTH OF T. BRUCEI ON CHINESE HAMSTER LUNG CELLS

Day	Cell Numbers [a] (trypanosomes/ml)
0	1.5×10^5
1	4.0×10^5
2	1.2×10^6
3	2.1×10^6
4	1.9×10^6
5	1.2×10^6
6	1.6×10^6

a - One-third of the RPMI medium was changed daily.

TABLE III

GROWTH OF T. BRUCEI EATRO 110

DAY	Mean Cell Number[a] (trypanosomes/ml)	
	Chinese Hamster Lung	Buffalo Lung
1	2.0×10^5	2.6×10^5
2	7.8×10^5	1.5×10^6
3	3.6×10^6	4.0×10^6
4	3.3×10^6	3.7×10^6

a – One-third of the RPMI medium was changed daily. The counts presented are those before the culture was changed and each represents three replicate samples per day.

TABLE IV

GROWTH OF T. RHODESIENSE ON CHINESE HAMSTER LUNG CELLS

Day	Cell Numbers [a] (trypanosomes/ml)
0	1.0×10^5
1	4.5×10^5
2	3.5×10^6
3	5.4×10^6
4	3.4×10^6
5	3.3×10^6

a - One-third of the RPMI medium was changed daily. The
    counts presented are those before the culture was
    changed and each represents three replicate samples
    per day.

TABLE V

RESPONSE OF SEVERAL TRYPANOSOME FORMS TO VARIOUS SUBSTRATES AND INHIBITORS

T. BRUCEI	α-GP STIMULATION	PROLINE STIMULATION	SHAM INHIBITION	AZIDE INHIBITION	AZIDE/SHAM INSENSITIVITY
Bloodstream Trypomastigotes	22	0	78	19	3
Cultured Infective Trypomastigotes	20	3	86	11	3
Procyclic Trypomastigotes	13	28	16	66	19

TABLE VI

INFECTIVITY OF T. RHODESIENSE GROWN ON

CHINESE HAMSTER LUNG CELLS

Trypanosomes	Inoculum of Trypanosomes	Days After Inoculum Before Infection Developed[a]
Cultured Infective Trypanosomes	1×10^6	4
Cultured Infective Trypanosomes	1×10^5	5
Bloodstream Stabilates	1×10^6	4
Bloodstream Stabilates	1×10^5	5

[a]The T. rhodesiense trypanosomes had been maintained on Chinese hamster lung tissue culture cells for 60 days. The infectivity tests are the results of two different experiments with six mice in each experiment.

TABLE VII

Protocol for Drug Treatment Experiments with *T. brucei*

Drug	Amount mg/kg	Parasitemia Level at Treatment cells/ml	Parasitemia Level at Sacrifice cells/ml	Length of Treatment hours
Berenil	10	1.0×10^9	6.0×10^8	$1\frac{1}{2}$
Suramin	10	9.3×10^8	3.2×10^8	$12\frac{1}{2}$
Antrycide	10	8.4×10^8	2.0×10^8	$12\frac{1}{2}$
Ethidium Bromide	10	1.0×10^9	7.8×10^8	2
Control	0	1.2×10^9	2.0×10^9	---

TABLE VIII

Inhibition of ^{3}H-Uridine Incorporation in T. brucei EATRO 110 In Vitro

Drug	Percent Inhibition
Ethidium Bromide	48.2 ± 4.2
Antrycide	81.4 ± 3.3
Berenil	87.2 ± 3.7
Suramin	88.3 ± 2.5

Infected rats were treated with the trypanocidal drugs at 10 mg/kg. The trypanosomes were isolated at various times as noted in Table V and used to study RNA synthesis. The percent inhibitions presented are the average of three different experiments. Standard errors of these results are also given.

TABLE IX

Inhibition of ^{3}H-Leucine Incorporation
in T. brucei EATRO 110 In Vitro

Drug	Percent Inhibition
Suramin	0.2 ± 1.5
Ethidium Bromide	14.2 ± 2.0
Berenil	30.1 ± 2.3
Antrycide	64.2 ± 3.2

Infected rats were treated with the trypanocidal drugs at 10 mg/kg. The trypanosomes were isolated at various times as noted in Table V and used to study protein synthesis. The percent inhibitions presented are the average of three different experiments. Standard errors of these results are also given.

TABLE X

Inhibition of RNA Synthesis in T. brucei EATRO 110
by Actinomycin

Drug Concentration (μM)	Percent Inhibition	
	Bloodstream Trypomastigotes	Procyclic Trypomastigotes
3.6	87	52
7.2	89	64
35.6	91	92

TABLE XI

EFFECT OF TRYPANOCIDAL DRUGS ON THE RESPIRATION OF T. BRUCEI

Drug	Percent Change in Respiration in Comparison to Control
Control	0
Suramin	+110.3 ± 6.0
Antrycide	+ 6.1 ± 0.8
Ethidium Bromide	− 0.5 ± 0.1
Berenil	− 31.2 ± 2.4

The respiration was measured in the open steady state system we have previously described (12). Infected rats were treated with the trypanocidal drugs at 10 mg/kg. The trypanosomes were isolated at various times as noted in Table and used for respiratory studies. The differences expressed in comparison to controls represent the averages of three different experiments. Standard errors from these experiments are also given.

TABLE XII

Analysis of Amino Acids in SM Medium Before and After
Growth of Trypanosoma rhodesiense EATRO 1895

Amino Acid	Per Cent of Total Detectable Amino Acids		
	Day Zero	Day 7	Percent Change
Lysine	2.25	1.03	−1.22
Tyrosine	0.28	0.07	−0.21
Glutamine + Glutamic Acid	1.40	2.76	+1.36
Aspartic Acid	5.96	1.00	−4.96
Phenylananine	2.83	4.13	+1.30
Methionine	0.59	0.65	+0.06
Serine	0.41	0.66	+0.25
Proline	28.89	45.60	+16.71
Leucine	36.00	26.20	−9.40
Isoleucine	0.45	0.26	−0.19
Glycine	0.70	0.99	+0.29
Valine	1.71	1.76	+0.05
Alanine	7.61	13.21	+5.60

Figure Legends

Fig. 1. The site of action of various inhibitors on the nucleo-cell sap
system and the mitochondrial system.

Fig. 2. T. brucei cultured infective trypomastigotes growing in the spaces
between the buffalo lung tissue culture cells. The cells had been growing
in culture for 25 days. The photograph of the trypanosomes on tissue culture
cells were taken while they were alive. X200.

Fig. 3. Growth of T. brucei EATRO 110 on Chinese hamster lung tissue
culture cells. The trypanosomes had been growing in vitro for 25 days.
The trypanosomes are growing close to the tissue culture cells. X200.

Fig. 4. T. rhodesiense cultured infective trypomastigotes growing on the
Chinese hamster lung cells. The cells were free-swimming in the medium
and had been growing in culture for 25 days. X200.

Fig. 5. T. rhodesiense cultured infective trypomastigotes growing in the
spaces between the tissue culture cells. The cells had been in culture
for 25 days. X200.

Fig. 6. A and B. Giemsa stain of T. brucei. Note the subterminal position
of the kinetoplast. The trypanosomes had been growing on buffalo lung
tissue culture cells for 25 days. X1000.

Fig. 7. Electron micrograph of T. rhodesiense which was grown on Chinese hamster
lung tissue culture cells for 60 days. Note the presence of the surface
coat (SC), plasma membrane (pm), mitochondrion (M) and the microtubules
(mt). The surface coat also surrounds the flagellum (F). X100,000.

Fig. 8. Electron micrograph of T. rhodesiense which was grown on Chinese
hamster lung tissue culture cells for 60 days. The mitochondrion (M)
has few cristae. Note the presence of the surface coat (SC) and the
microbody (mb). X45,000.

Fig. 9. Electron micrograph of T. rhodesiense cultured infective trypomasti-
gotes which had been maintained on Chinese hamster lung tissue culture cells
for 80 days. The surface coat (SC), plasma membrance (pm) and microtubules
(mt) are very distinct. X100,000.

Fig. 10. Electron micrograph of T. rhodesiense which was grown on Chinese
hamster lung cells for 80 days. Note the presence of the surface coat. X100,000.

Fig. 11. Electron micrograph of T. rhodesiense slender bloodstream trypomasti-
gotes. The surface coat is pronounced. (sc) surface coat; (pm) plasma
membrane; (mt) microtubules; (F) flagellum. X100,000.

Fig. 12. Electron micrograph of T. brucei procyclic trypomastigote. Note the absence of the surface coat. (N) nucleus; (F) flagellum; (K) kinetoplast; (M) mitochondrion. X24,000.

Fig. 13. Electron micrograph of T. brucei bloodstream trypomastigotes. Note the presence of the surface coat. (K) kinetoplast. X24,000.

Fig. 14. Electron micrograph of T. brucei bloodstream trypomastigotes. Note the presence of the surface coat. X60,000.

Fig. 15. Steady state oxygen trace of T. rhodesiense bloodstream trypomastigotes. The cell concentration was 1 x 10^8 cells.

Fig. 16. Steady state oxygen trace of T. rhodesiense cultured infective trypomastigotes maintained in vitro for 60 days on Chinese hamster lung tissue culture cells. The concentration was 1.0 x 10^8 cells.

Fig. 17. Steady state oxygen trace of T. rhodesiense procyclic trypomastigotes. The concentration of cells was 8.0 x 10^7 cells.

Fig. 18. Graph of infectivity test of cultured trypomastigotes of T. brucei. The control was a stabilate of T. brucei bloodstream forms. The cultured trypomastigotes had been maintained in vitro for 20 days on buffalo lung tissue culture cells.

Fig. 19. Electron micrograph of T. brucei EATRO 110 bloodstream trypomastigotes treated with berenil (10 mg/kg) for 90 minutes. A dyskinetoplastic trypanosome is present. X12,000.

Fig. 20. Electron micrograph of T. brucei EATRO 110 bloodstream trypomastigotes treated with berenil (10 mg/kg) for 90 minutes. There is a marked effect on the nuclear membrane and chromatin. X12,000.

Fig. 21. Electron micrograph of T. brucei EATRO 110 bloodstream trypomastigotes treated with berenil (10 mg/kg) for 90 minutes. This micrograph demonstrates the nucleus at several different stages of disruption or ultrastructure changes after treatment with berenil. X12,000.

Fig. 22. Effect of berenil on the growth of T. brucei in a rat. With treatment of berenil (5.0 mg/ml), trypanosomes are absent from the peripheral blood with 90 minutes.

Figs. 23 and 24. Electron micrographs of T. brucei EATRO 110 bloodstream trypomastigotes treated in vivo with ethidium bromide (10 mg/kg) for 2 hours. A dyskinetoplastic cell can be seen. X12,000.

Fig. 25. Electron micrograph of T. brucei EATRO 110 bloodstream trypomastigotes treated in vivo with ethidium bromide (10 mg/kg) for 2 hours. The nuclear membranes of the cells present show some effect of the drug. X12,000.

Fig. 26. Electron micrograph of T. brucei EATRO 110 bloodstream trypomastigotes treated in vivo with antrycide (10 mg/kg) for 12.½ hours. X12,000.

Fig. 27. T. brucei EATRO 110 bloodstream trypomastigotes treated in vivo with suramin (10 mg/kg) for 12½ hours. Little or no effect on the ultra-structure of the trypanosomes was observed. X12,000.

Fig. 28. T. brucei EATRO 110 trypomastigotes which were not treated with any drug. These cells represent the controls for the experiment discussed in Table VII. X12,000.

Fig. 29. Inhibition of ^{3}H uridine incorporation in T. brucei EATRO 110 bloodstream trypomastigotes exposed to trypanocidal drugs in vivo. The concentration of the drugs used in all cases was 10 mg/kg.

Fig. 30. Inhibition of ^{3}H leucine incorporation in T. brucei EATRO 110 bloodstream trypomastigotes exposed to trypanocidal drugs in vivo. The concentration of the drugs used in all cases was 10 mg/kg.

Fig. 31. Percent inhibition by ethidium bromide of RNA synthesis in T. brucei EATRO 110 bloodstream and procyclic trypomastigotes.

Fig. 32. Growth of T. rhodesiense in normal and modified SM medium.
(Δ——Δ) cells grown in SM medium (25/ml/140/ml volume);
(O——O) cells grown in modified SM medium (25 ml/140/ml volume);
(☐——☐) cells grown in modified SM medium (350 ml/855/ml volume).

Fig. 33. Procedure for T. rhodesiense DNA-cellulose ^{32}P-RNA hybridization columns.

Figs. 34 and 35. Banding of cytoplasmic ribosomes from T. brucei procyclic trypomastigotes. The ribosomes were isolated in 50 mM Mg Cl$_2$ in TKM buffer in a gradient of 15 - 30% sucrose. The sample was spun for 18 hours at 4°C at 24,000 rpm. Some breakdown was observed when 10 mM Mg Cl$_2$ was used (Figure 35).

Fig. 36. Transformation experiment with T. brucei Lump 1026. The initial inoculum was from a rat at 2.1 x 10^8 cells/ml and a day 8 parasitemia. The trypanosomes had completely transformed from bloodstream to procyclic trypomasti-gotes within 72 hours.
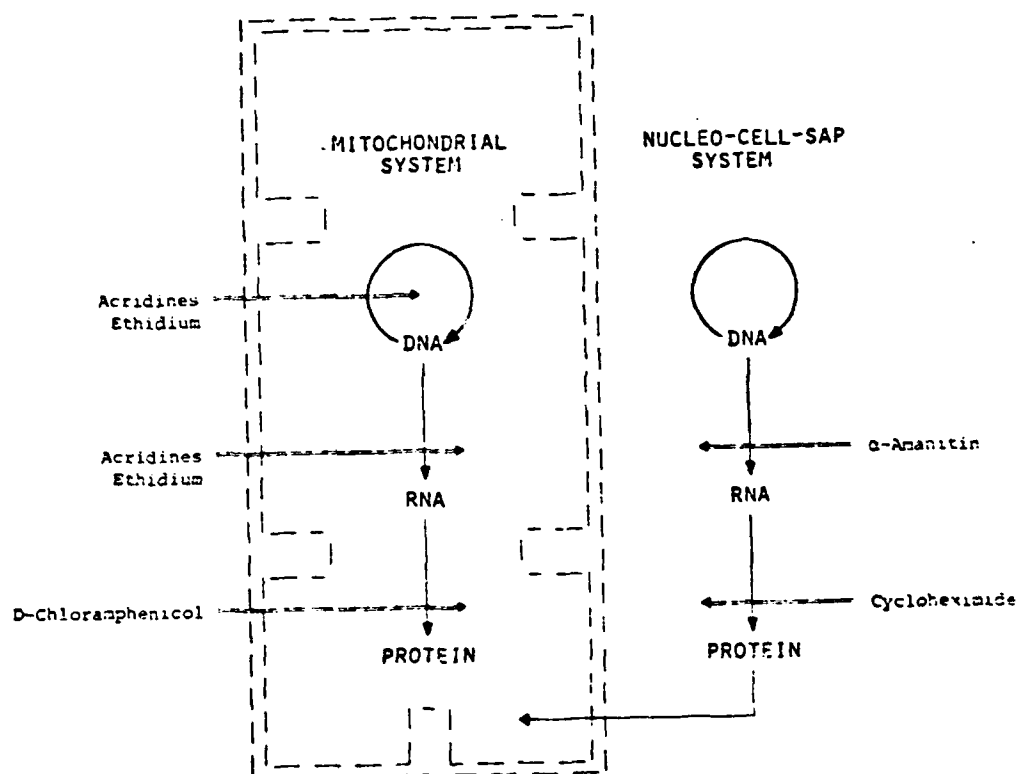
Figure 1

Figure 2

Figure 3
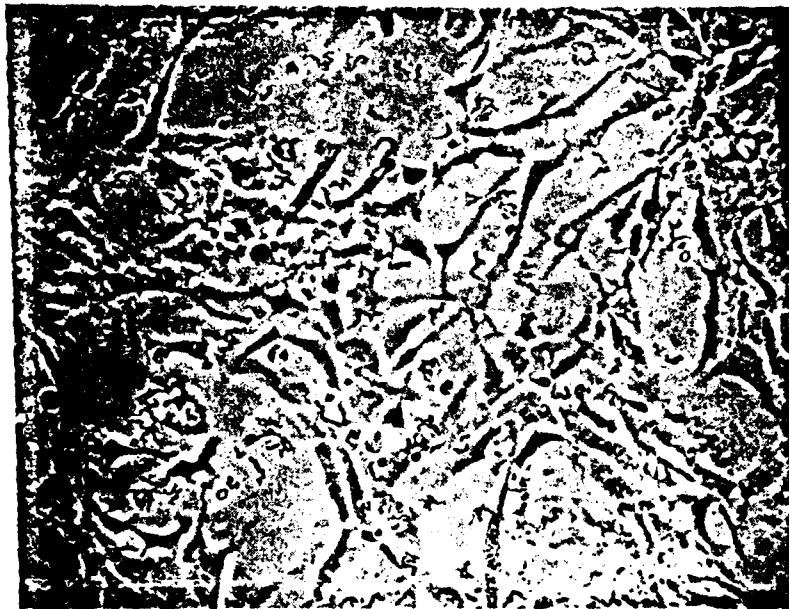
Figure 4

Figure 5

Figure 6A

Figure 6B
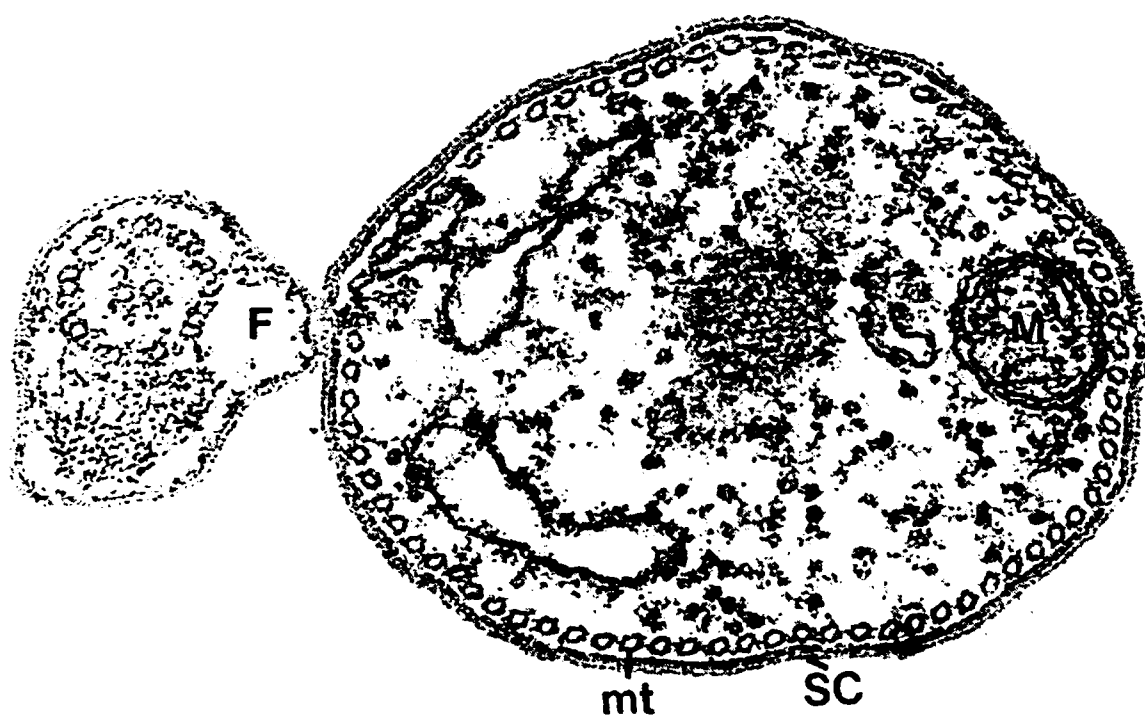
Figure 7
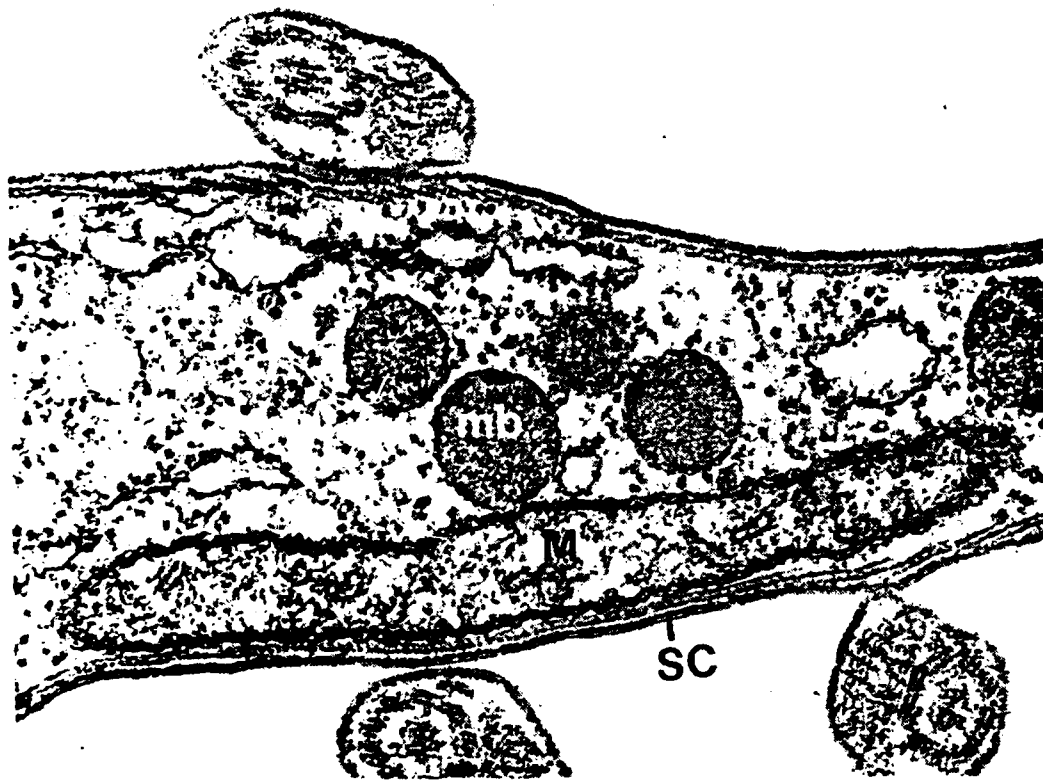
Figure 8
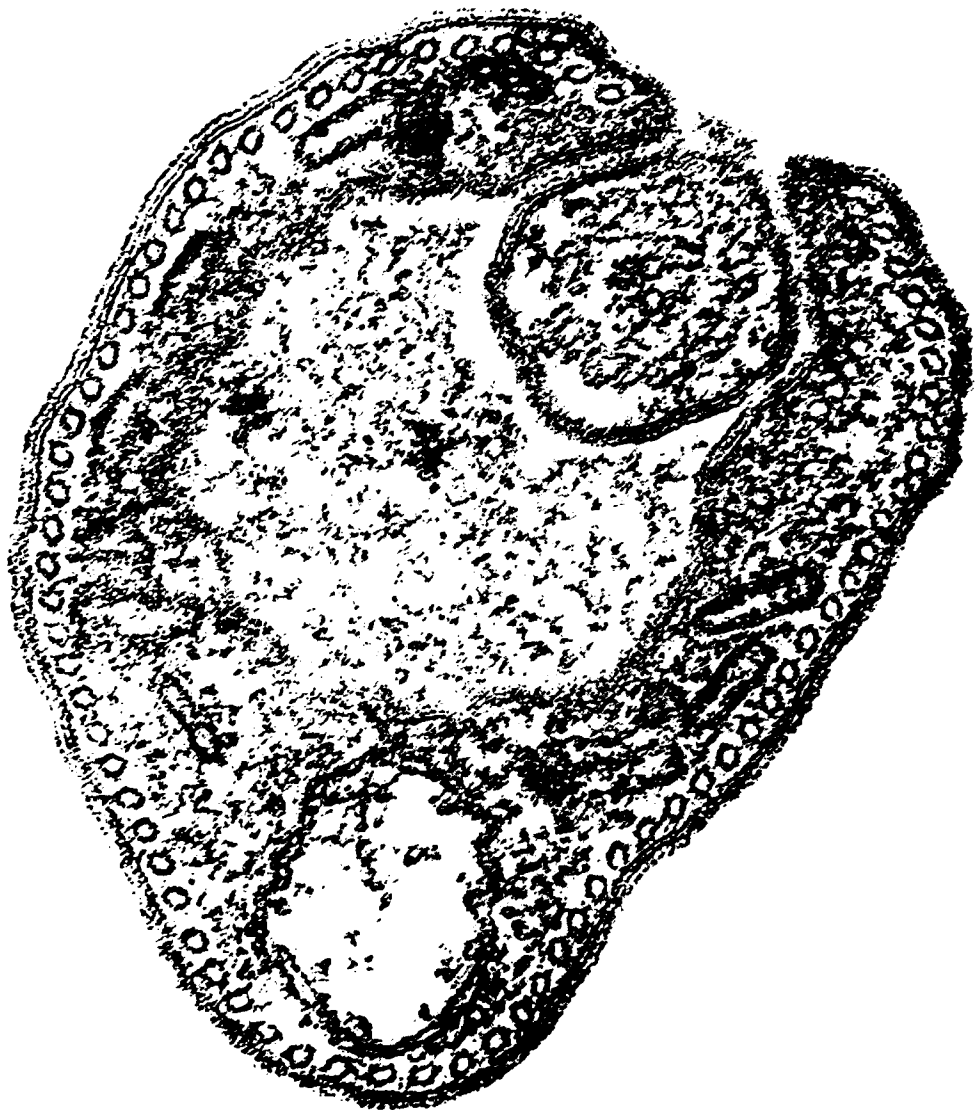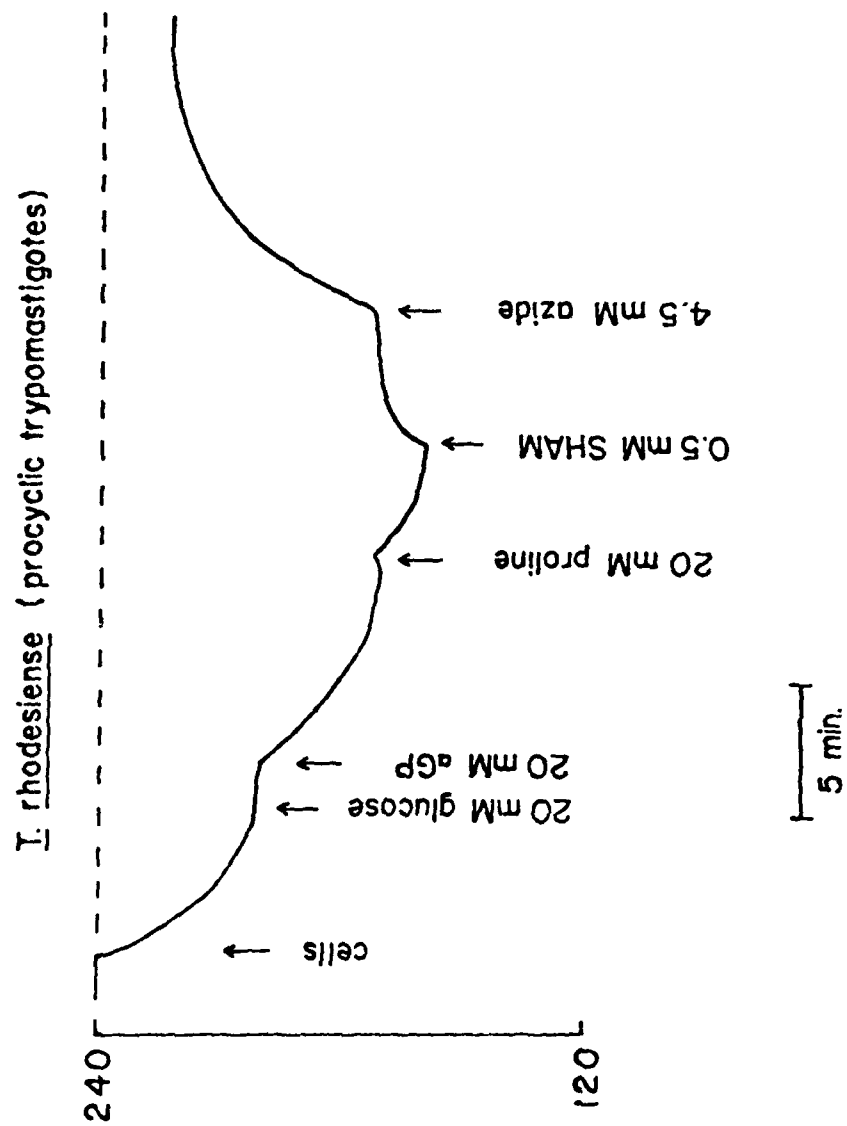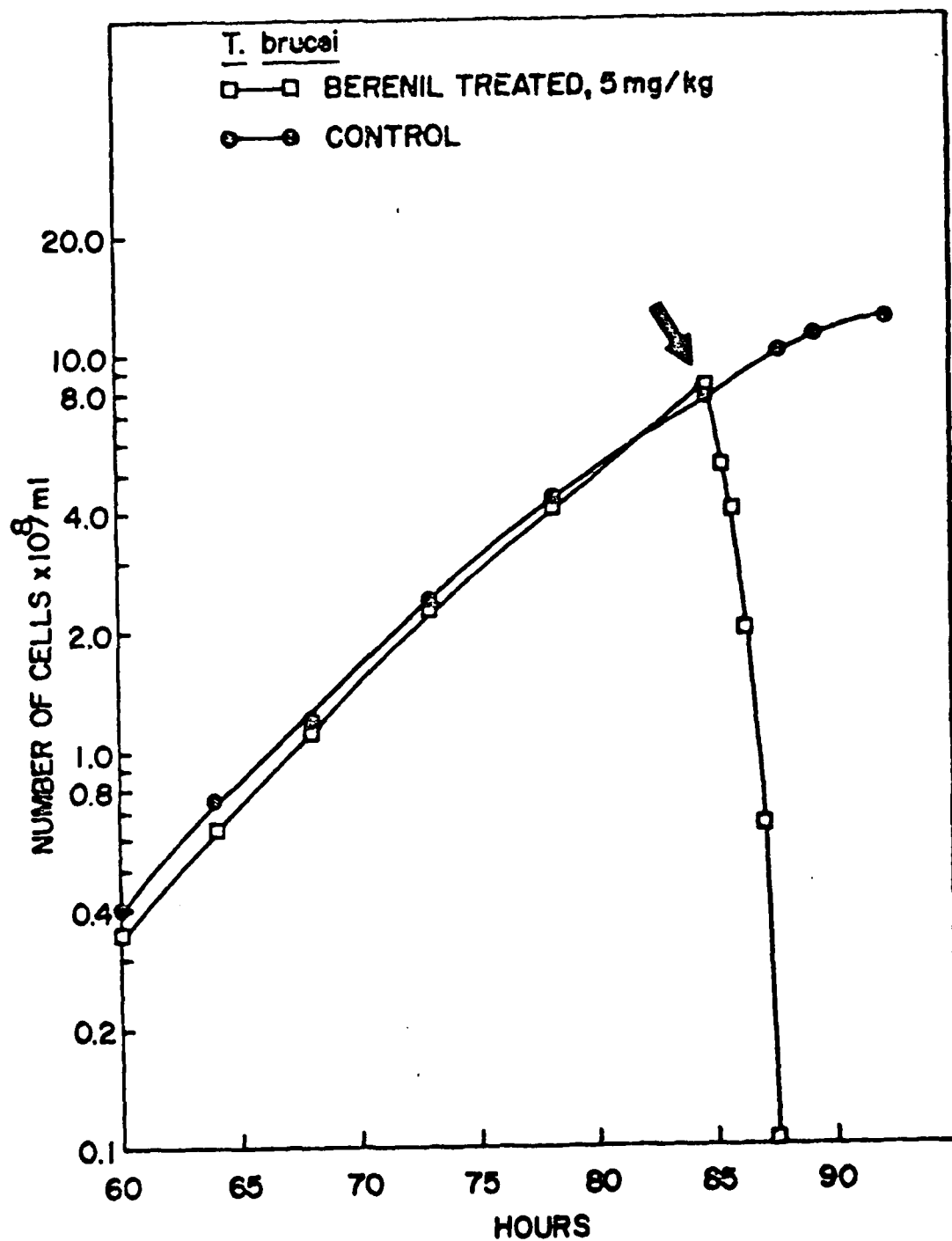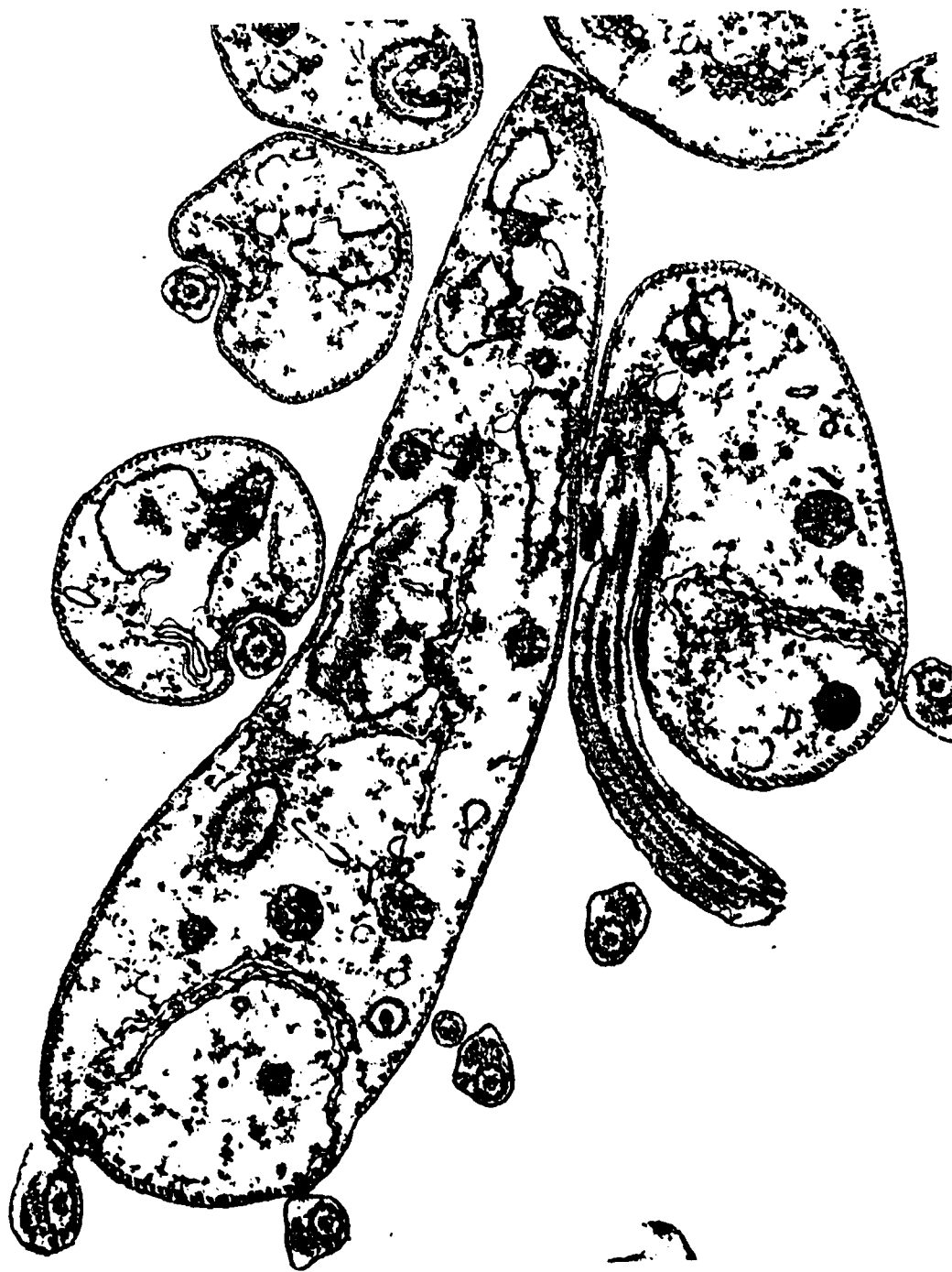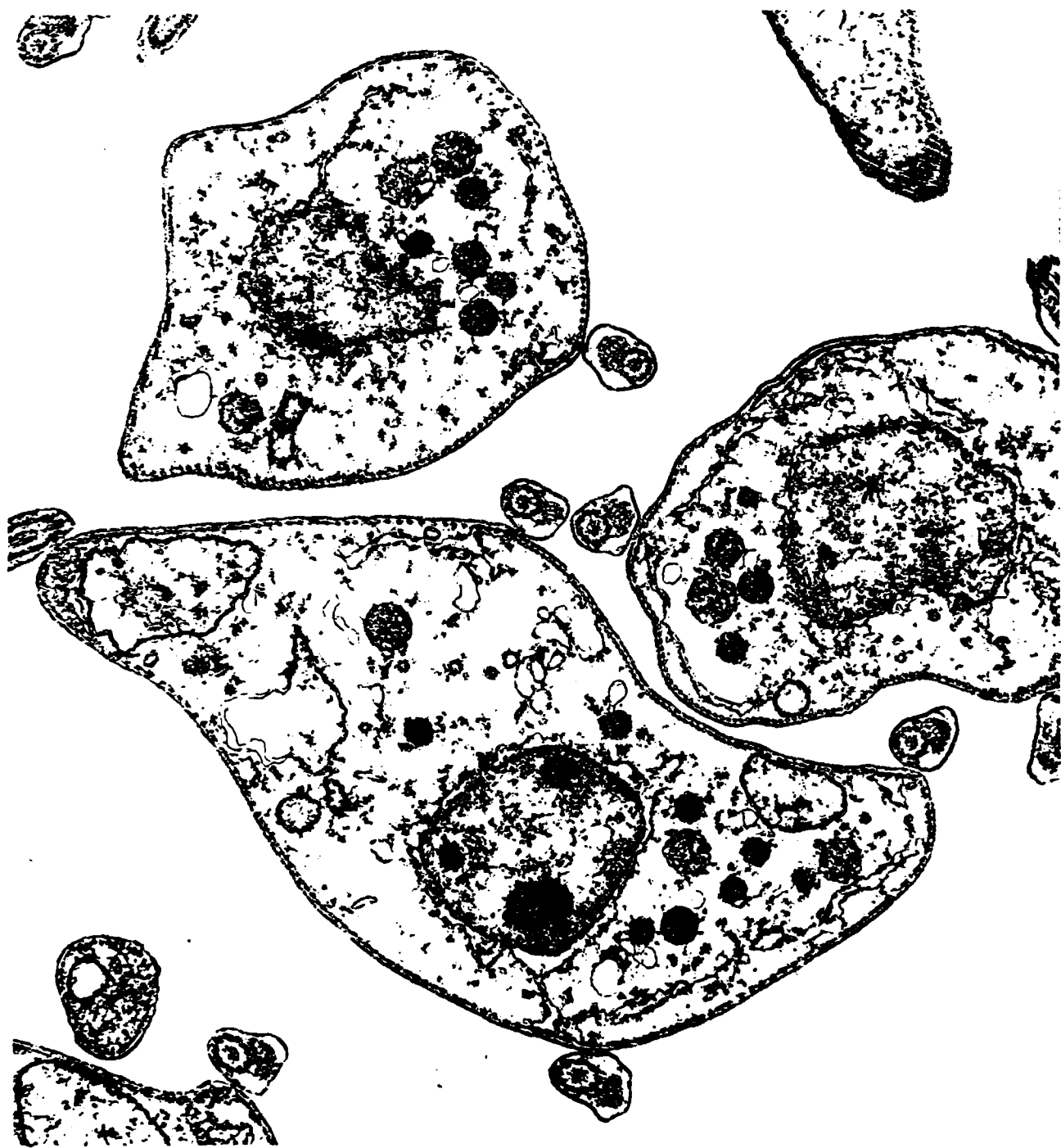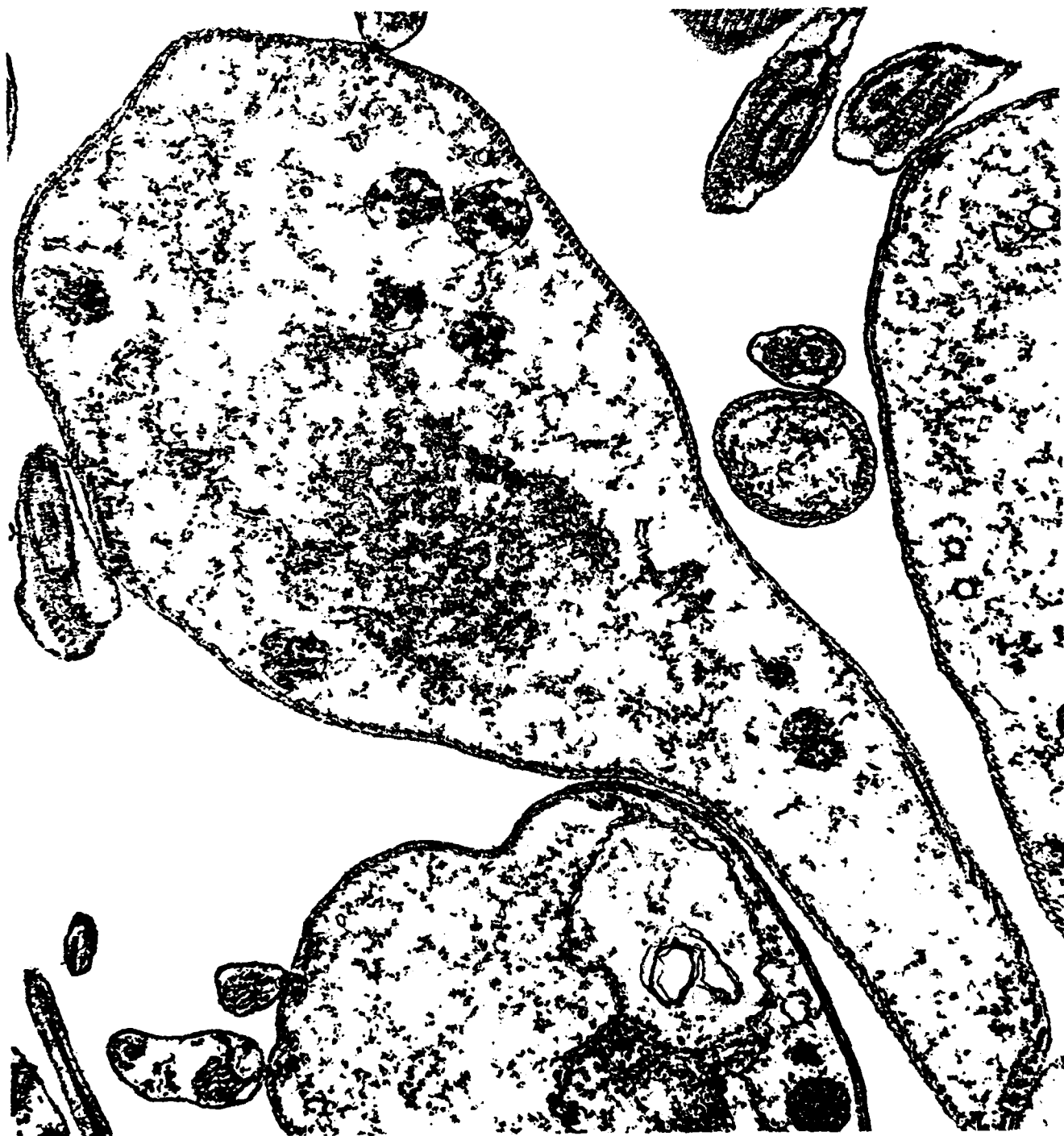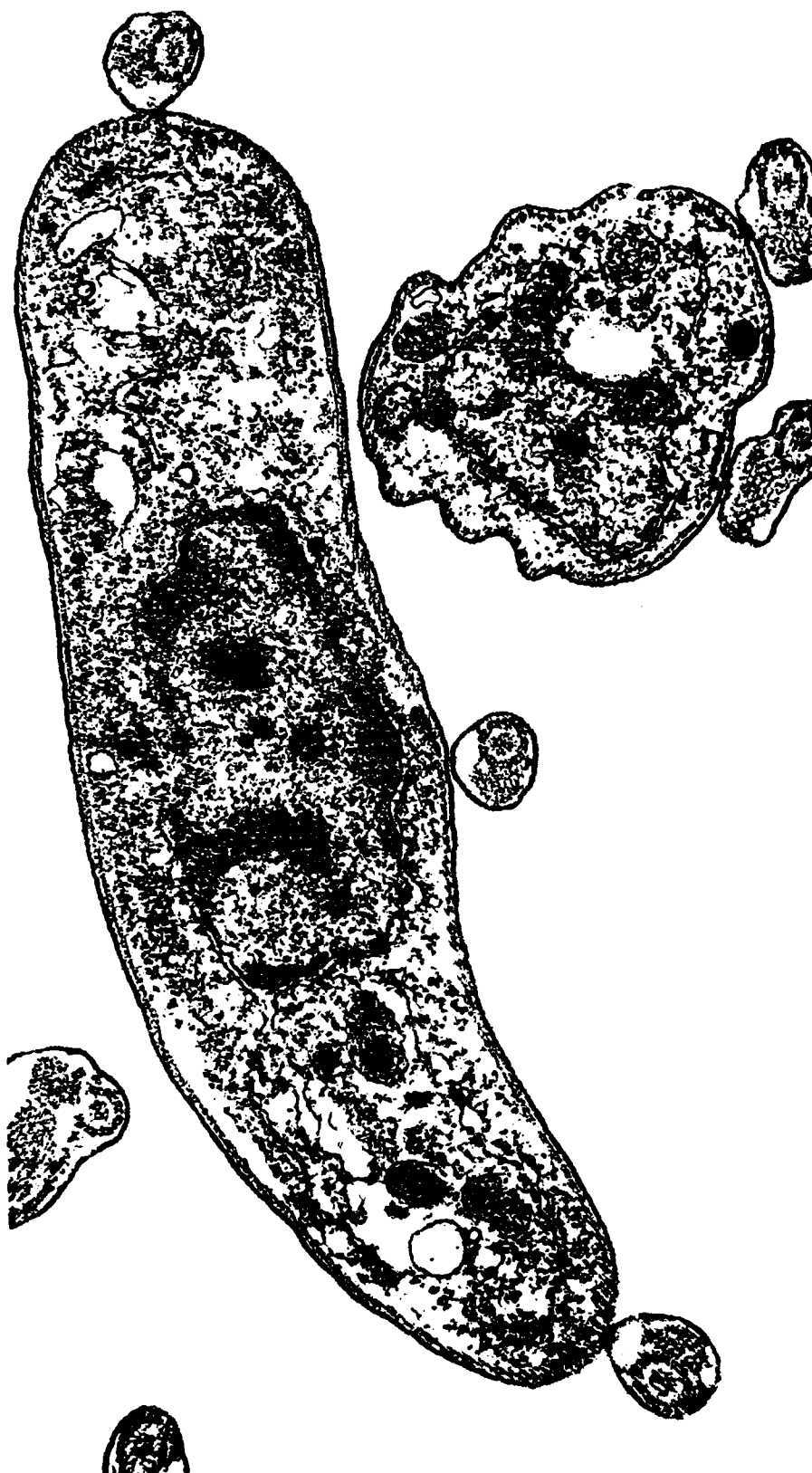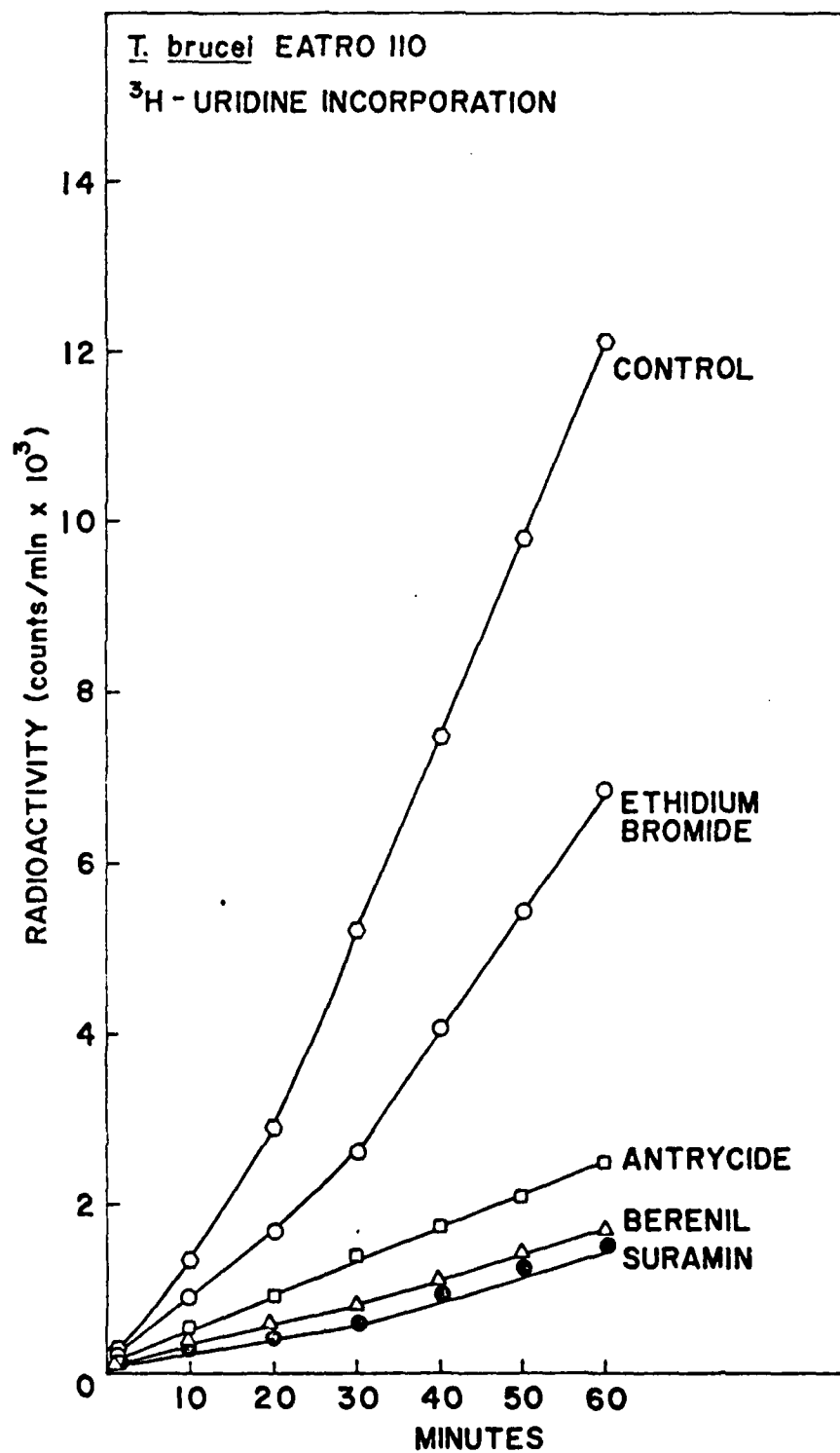
Figure 9
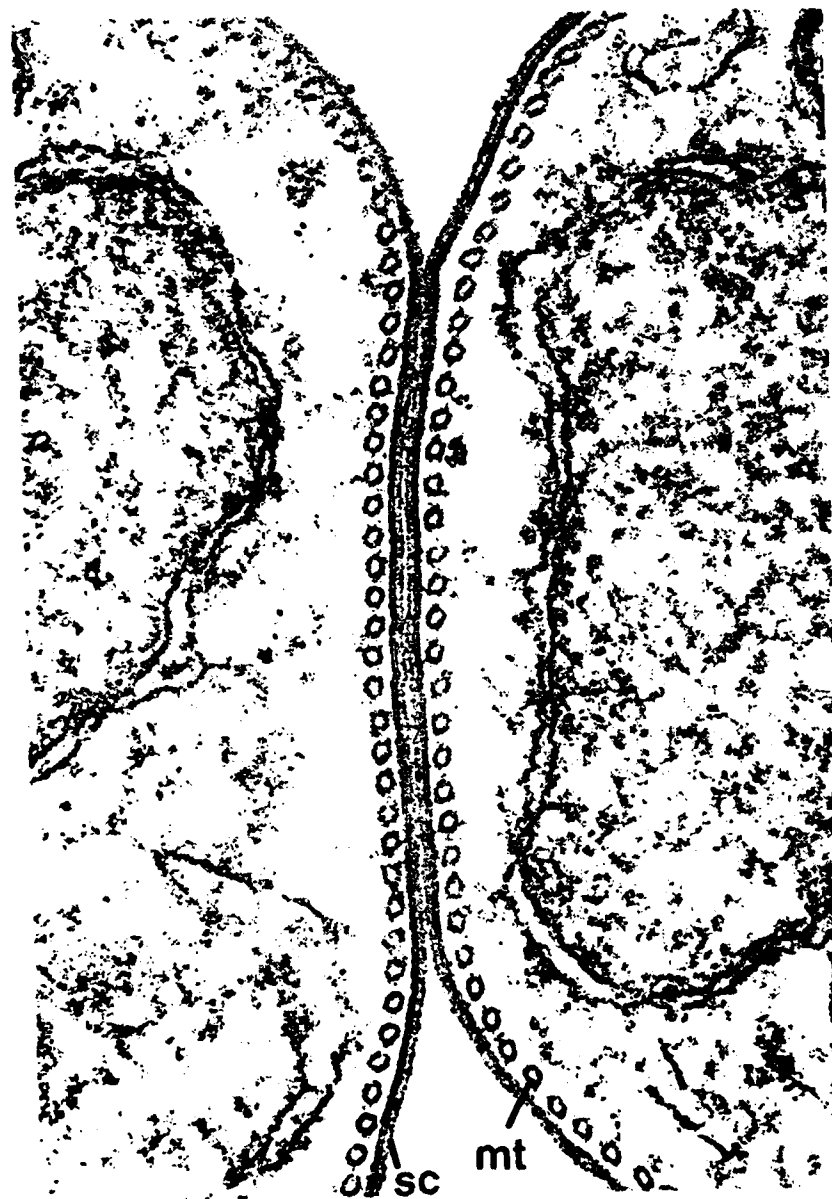
Figure 10
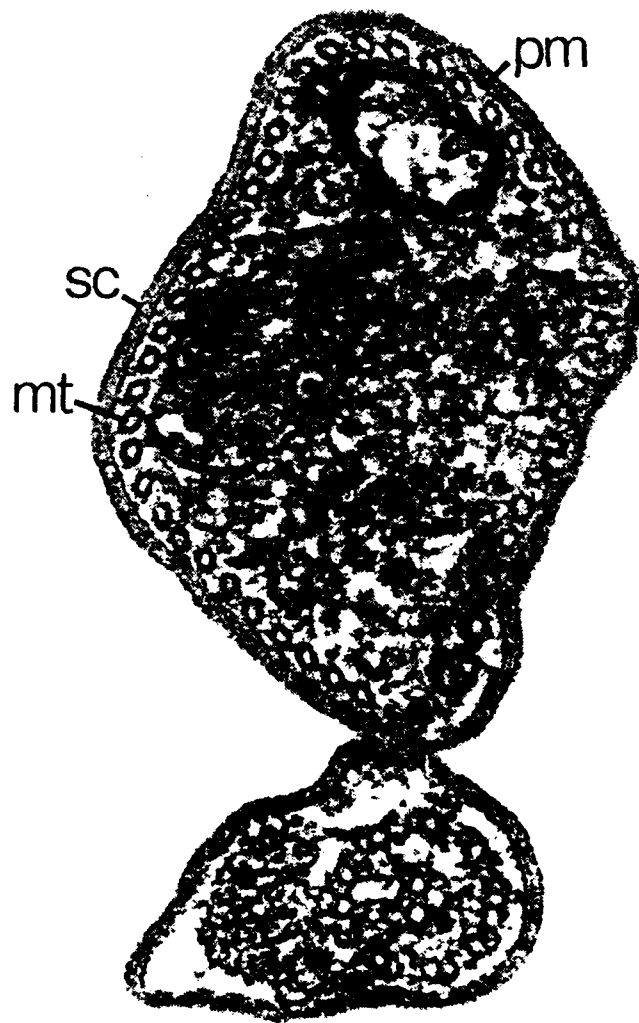
Figure 11

Figure 12

Figure 13

Figure 14

T. rhodesiense (bloodstream trypomastigotes)

240

120

← cells

← 20 mM glucose

← 20 mM αGP

5 min.

← 20 mM proline

← 4.5 mM azide

← 0.5 mM SHAM

Figure 15

T. rhodesiense (cultured infective trypomastigotes)

240

120

cells

20 mM glucose

20 mM αGP

20 mM proline

4.5 mM azide

0.5 mM SHAM

5 min.

Figure 16

T. rhodesiense (procyclic trypomastigotes)

cells

20 mM glucose
20 mM αGP

20 mM proline

0.5 mM SHAM

4.5 mM azide

240

120

5 min.
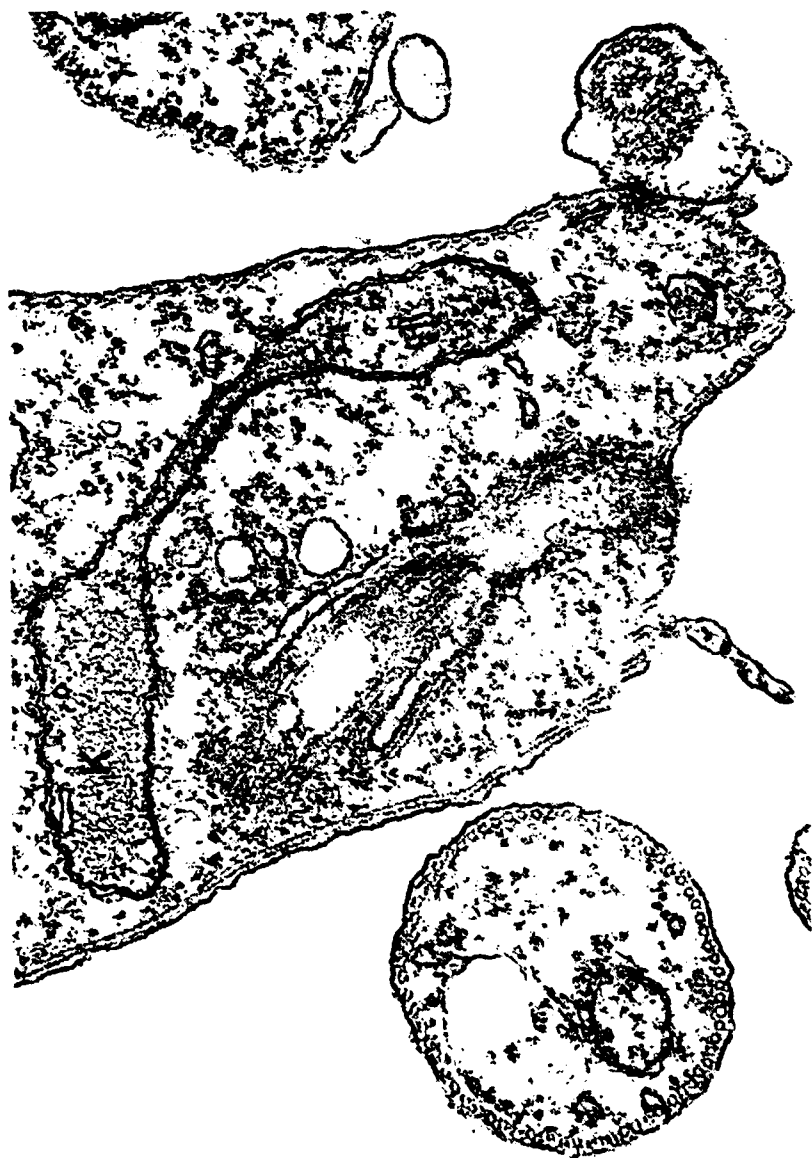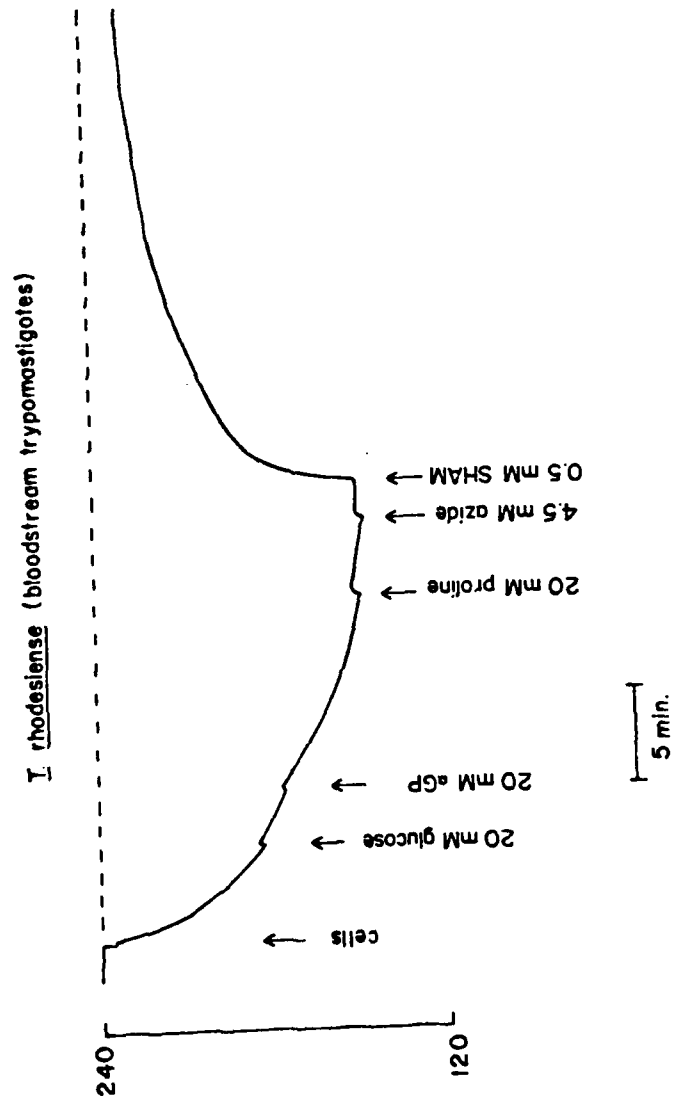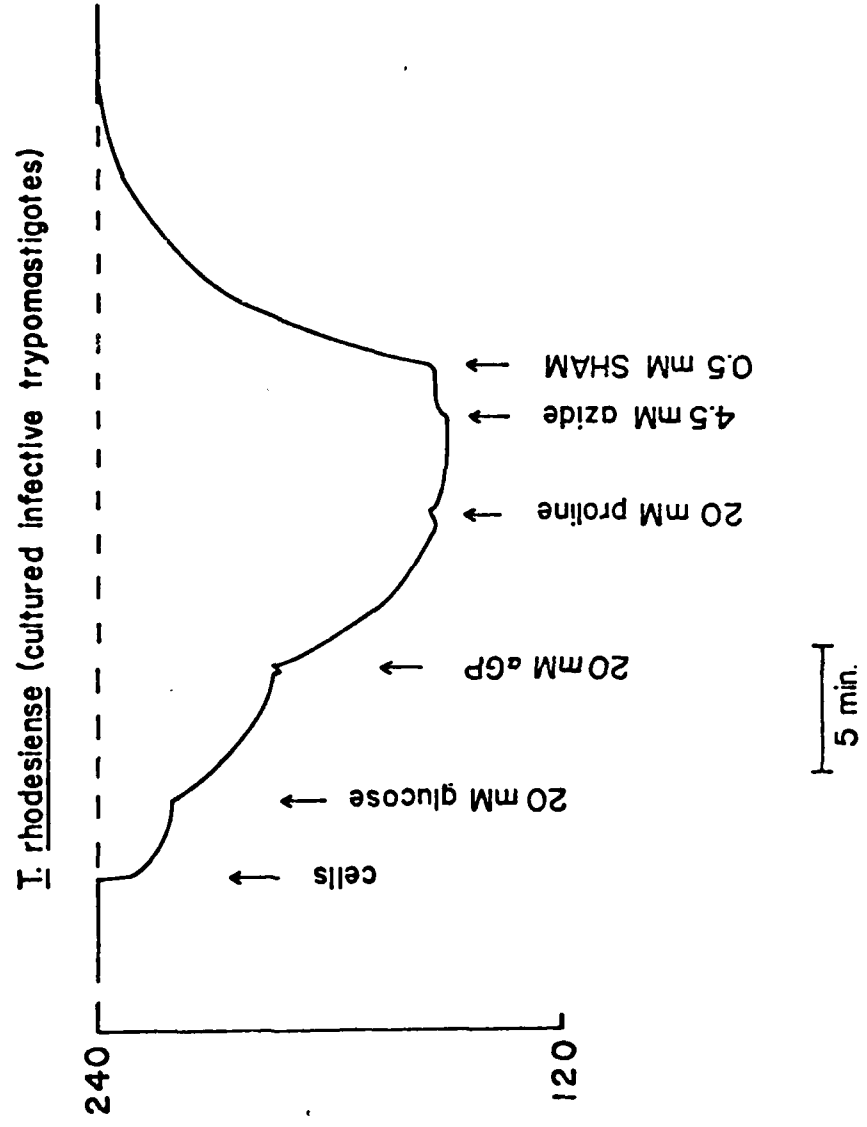
Figure 17

INFECTIVITY STUDIES OF CULTURED TRYPOMASTIGOTES
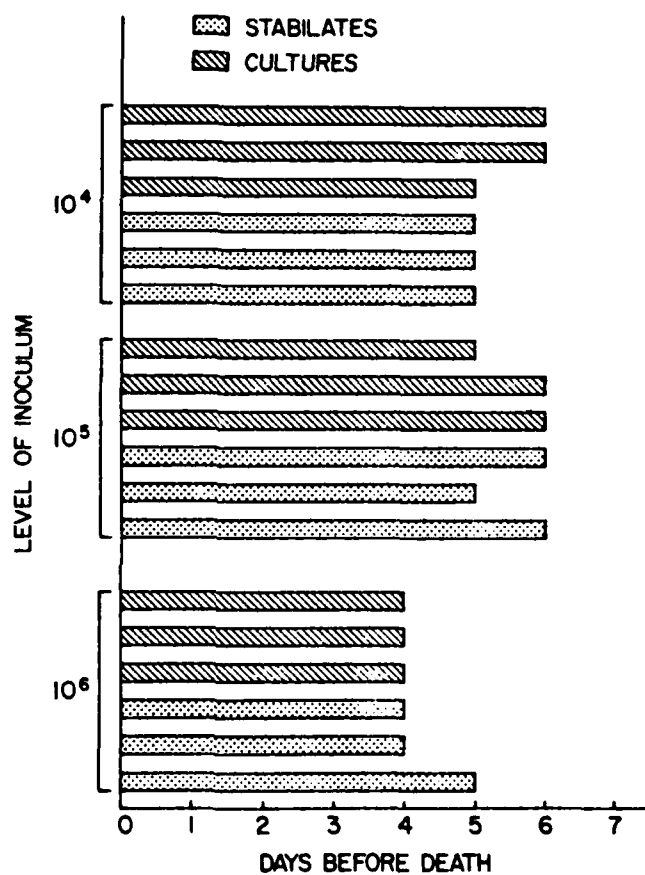
Figure. 18

Figure 19

Figure 20

Figure 21

Figure 22

Figure 23

Figure 24

Figure 25

Figure 26

Figure 27

Figure 28

Figure 29

T. _brucei_ EATRO 110
^{3}H – LEUCINE INCORPORATION

CONTROL

ETHIDIUM BROMIDE

BERENIL

ANTRYCIDE

RADIOACTIVITY (counts/min x 10^3)

MINUTES
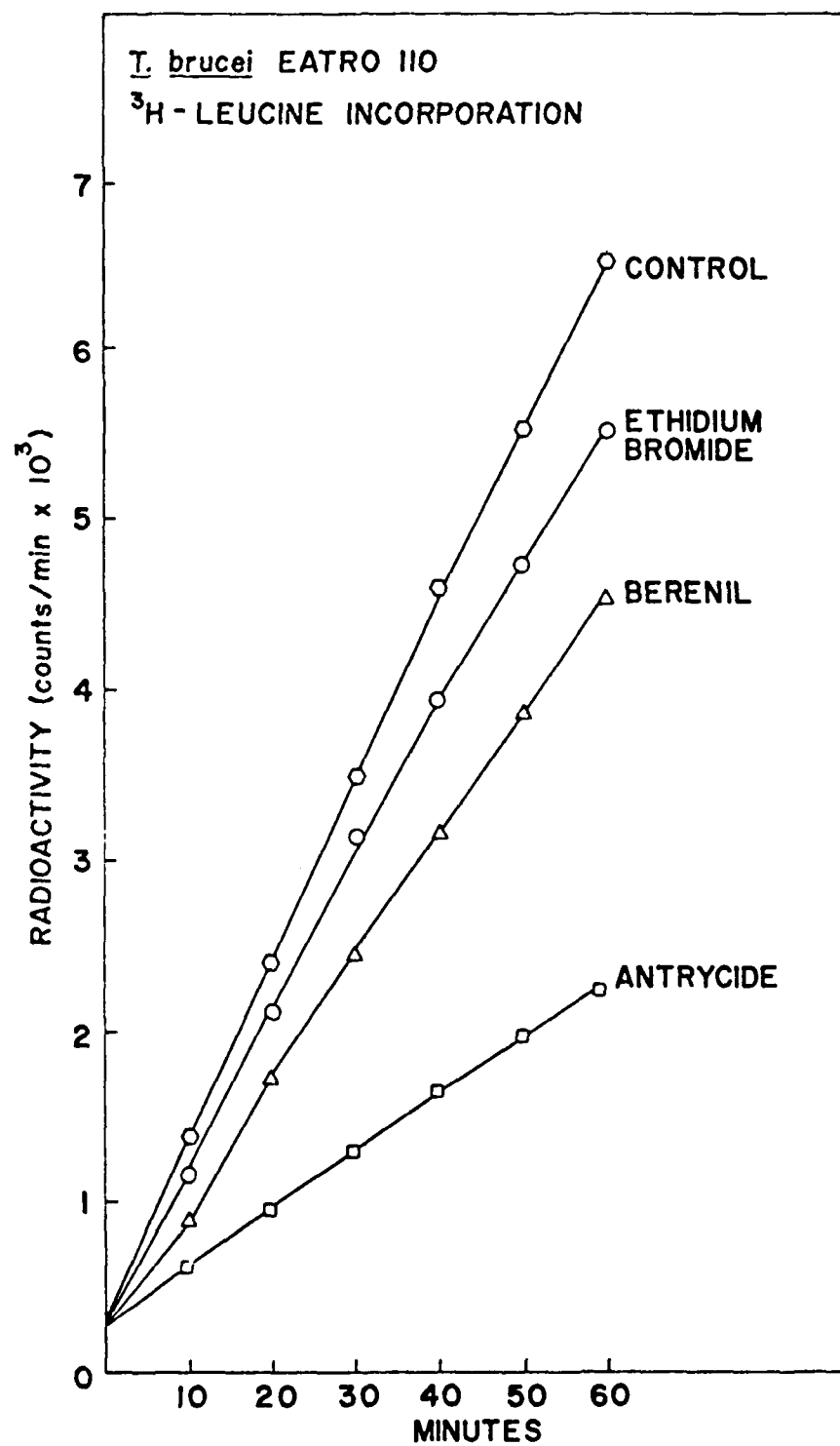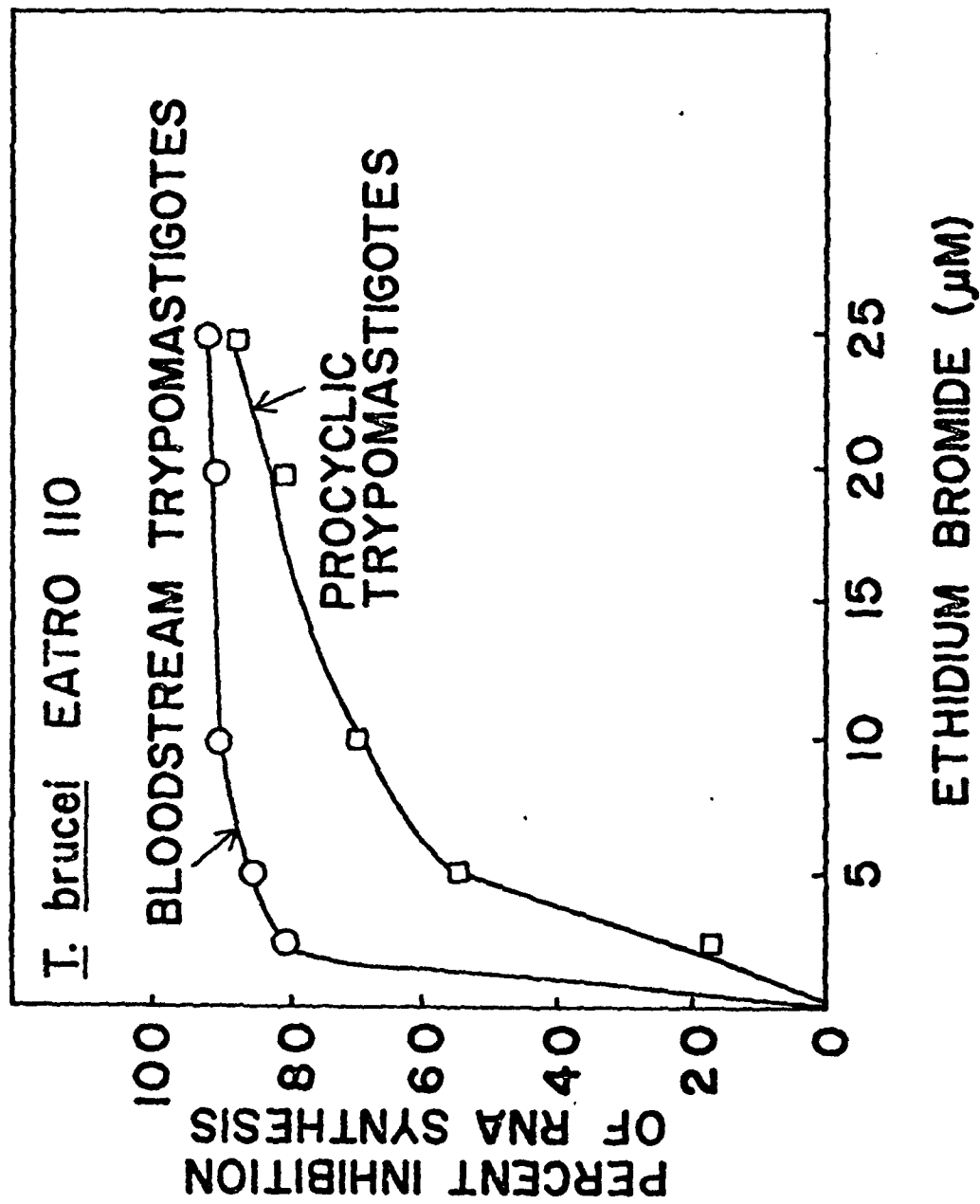
Figure 30

Figure 31

Figure 32

^{32}P - RNA

4°C

33°C
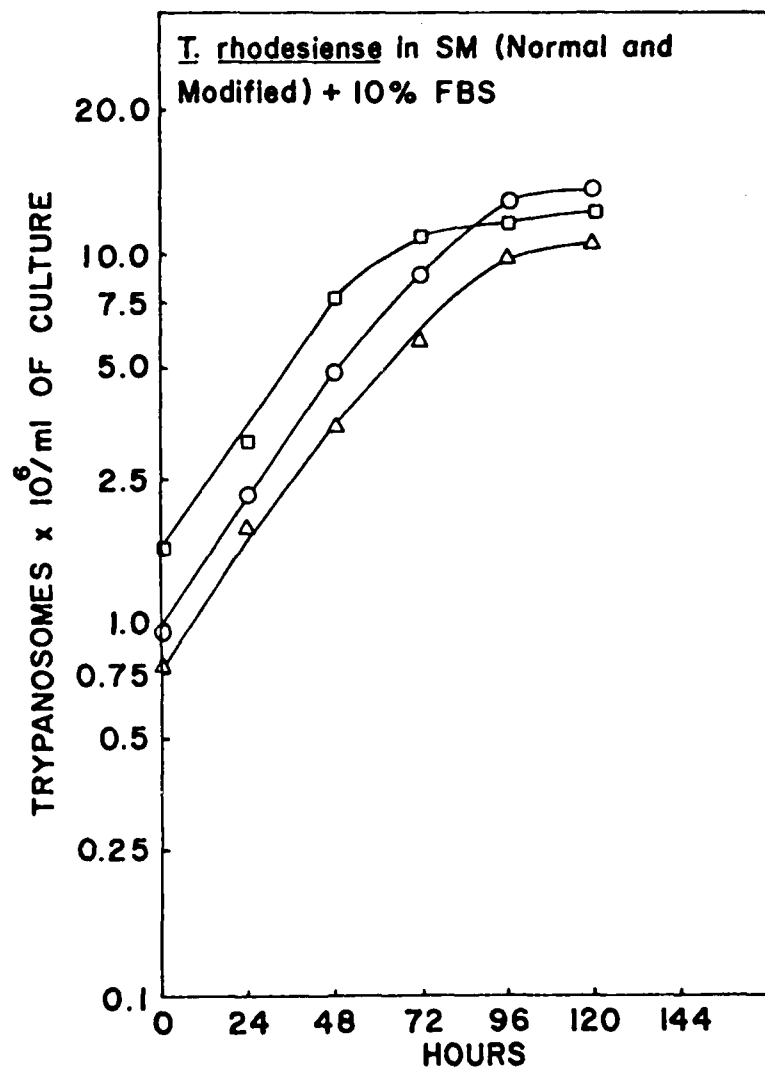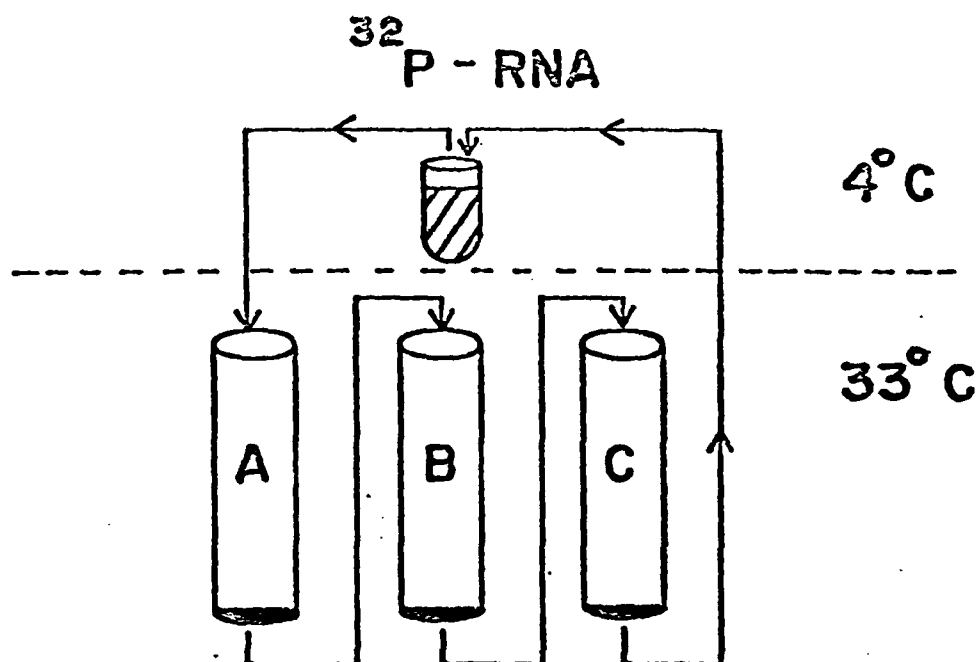
A = NUCLEAR DNA - RANDOM SEQUENCES

B = KINETOPLAST DNA - MINICIRCLE SEQUENCES

C = KINETOPLAST DNA - MAXICIRCLE SEQUENCES

Figure 33

Figure 34
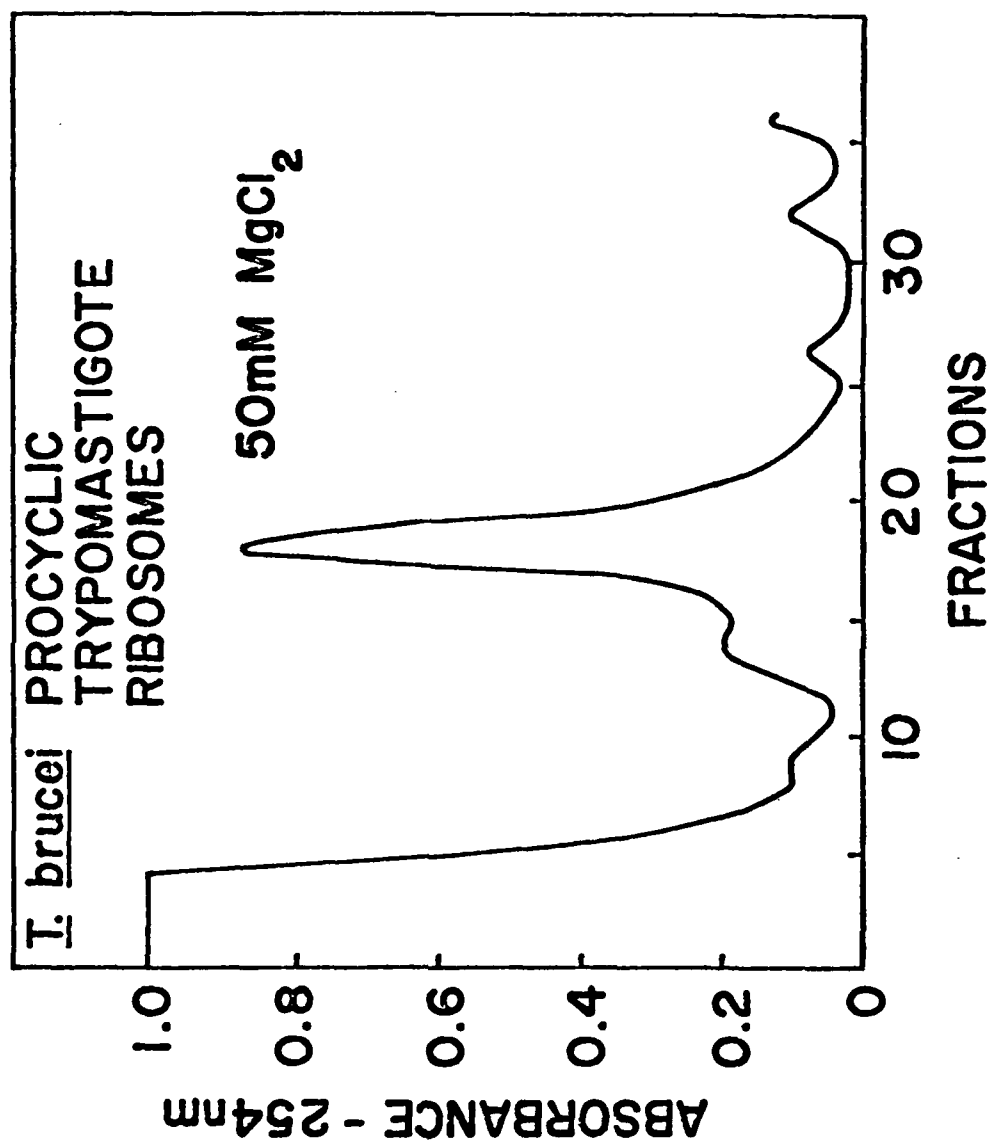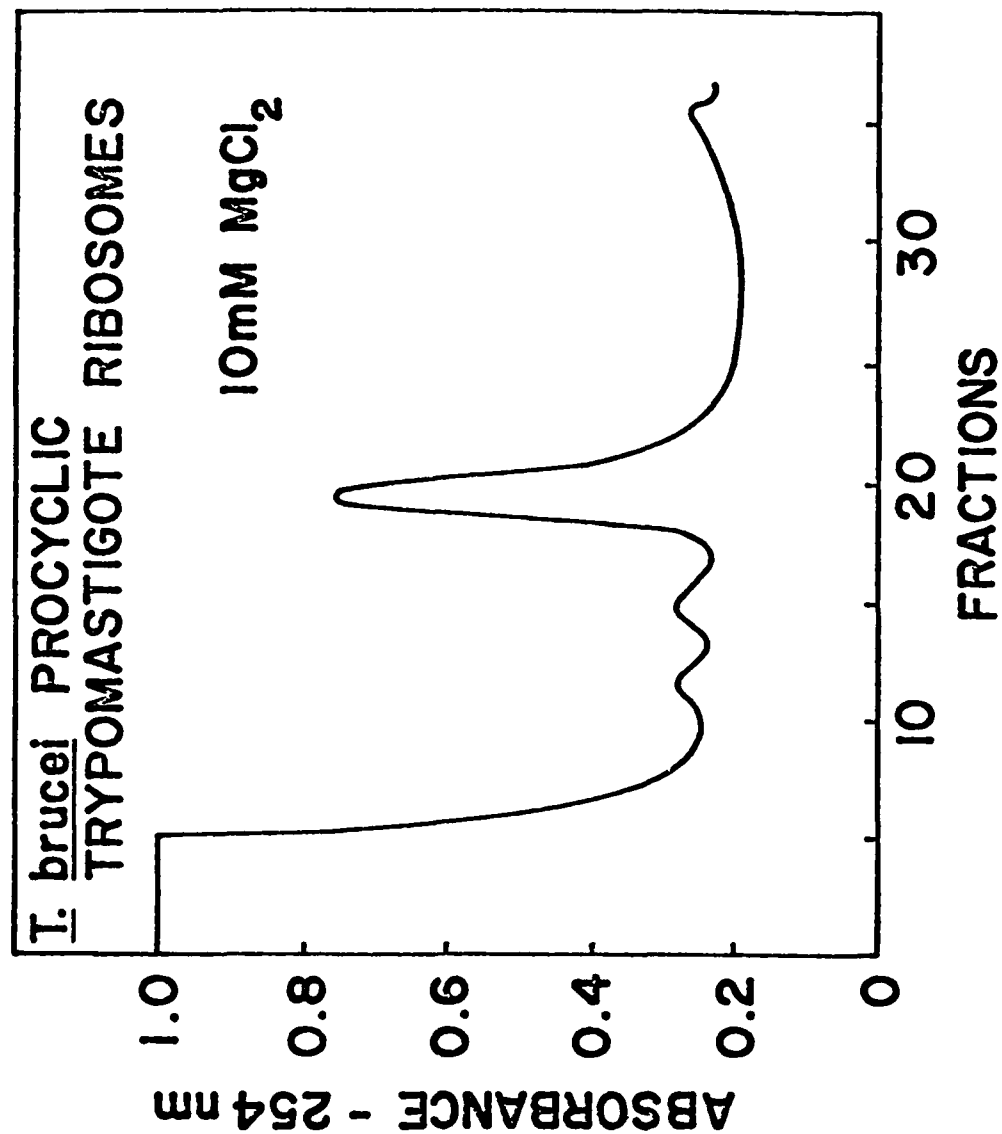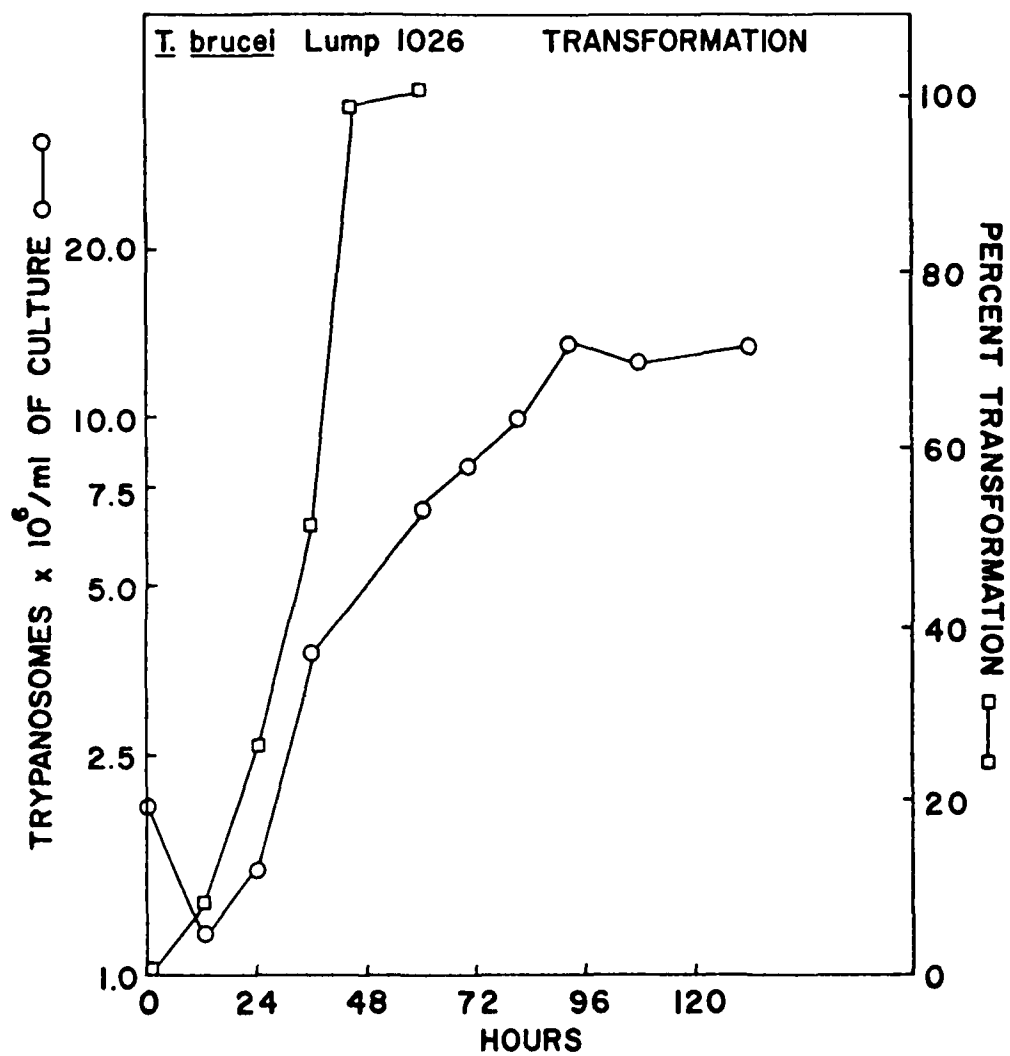
T. brucei PROCYCLIC TRYPOMASTIGOTE RIBOSOMES

10mM MgCl$_2$

ABSORBANCE - 254 nm

FRACTIONS

Figure 35

Figure 36